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Evolution and ecology of malaria parasites: from mating to mixed-species infections

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Abstract

Despite over a century of research, malaria parasites (*Plasmodium*) still remain a major cause of mortality and morbidity worldwide. In recent years, the application of theoretical principles from ecology and evolutionary biology to the study of these parasites has started to provide insight into variety of fundamental subjects from the evolution of virulence to the facultative strategies (i.e. phenotypic plasticity) that parasites use to maximize their transmission. It is now becoming increasingly clear that to understand and predict population level patterns of virulence and transmission, the processes that occur at the between-host level must be studied in light of the interactions that happen within hosts (between parasites and between parasites and hosts). In this thesis I combine concepts from evolutionary biology and ecology with tools from molecular and cellular biology and evolutionary genetics, which allow me to study rodent malaria parasites at both evolutionary and ecological timescales. The work I present in this thesis has the following four components:

1. Phylogenetics (chapter 2): I applied recently developed phylogenetic methods to a large DNA sequence dataset that I generated, to provide a better understanding of the phylogeny of rodent malaria parasites and investigate how selection has shaped their genomes. I show that all rodent malaria subspecies can be considered species, provide the first time line for the evolution of this group of parasites and demonstrate that most loci are under purifying selection.

2. Hybridization and reproductive isolation (chapter 3): I show that hybridization between two rodent malaria parasites (*P. berghei* and *P. yoelii*) can occur, but only occurs at high levels when one of two proteins (P230 or P48/45) is absent from the surface of female gametes, which indicates that these proteins are involved in gamete recognition. I find that P230, P48/45 and P47 (a possible interaction partner) are evolving under positive selection, a feature often observed in gamete recognition proteins of other taxa. Finally, I show that the fertilization success of *P. berghei* is reduced in the presence of *P. yoelii*, but not vice-versa, which indicates asymmetric reproductive interference.

3. Sex allocation (chapter 4): I carry the first test of sex allocation's assumption that immunity impacts on the fertility of *Plasmodium* male gametocytes/gametes more than on the fertility of females. I show that while the fertility of both males and females is equally affected, males are affected during gametogenesis and females are mostly affected through gamete dysfunction (i.e. gametes can mate but zygotes fail to develop), which is in agreement with the assumptions of theory. In collaboration, I incorporate these effects into sex allocation theory and predict that malaria parasites can minimize the effects of factors that kill gametocytes/gametes by adjusting their sex ratios. On the other hand sex ratio adjustment cannot compensate for gamete dysfunction or zygote death. These results have applied implications for transmission-blocking vaccines.

4. Infection dynamics of mixed-species infections (chapter 5): I develop a series of experiments to test how a focal parasite species (*P. yoelii*) is affected by competition with heterospecifics (*P. chabaudi*) and how the interaction between the two species is mediated by immunity and resource availability. I show that *P. chabaudi* can boost *P. yoelii* above its single species level (i.e. facilitation) and that this is mediated by resource availability. On the other hand, *P. yoelii*'s performance can also be hindered in mice that were exposed to a *P. chabaudi* infection. My results also reveal that host mortality is exacerbated in mixed-species infections of naïve mice, which may be due to an inability of the host to achieve the right balance between the production and the destruction of red blood cells, when dealing with a mixed-species infection.

The work I present here tackles fundamental questions concerning the transmission biology and the within-host interactions of malaria parasites The results presented demonstrate the importance of interactions between hosts and parasites and between different parasite species (at the molecular and the whole organism levels) for determining the outcome of transmission, virulence and within-host parasite performance.

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Appendices: Papers arising from this thesis

1. Reece, S. E., R. S. Ramiro, and D. H. Nussey. 2009. Plastic parasites: sophisticated strategies for survival and reproduction? . Evol Appl 2:11-23.

2. Ramiro RS, Alpedrinha J, Carter L, Gardner A, Reece SE (2011) Sex and Death: The Effects of Innate Immune Factors on the Sexual Reproduction of Malaria Parasites. PLoS Pathog 7(3): e1001309. doi:10.1371/journal.ppat.1001309

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Declaration

Science is a collaborative endeavor and a lot the of the work I present in this thesis would not have been possible without collaboration. To recognize this I keep the use of "we" in chapters 2-5. Chapters 1 and 6 were written solely as thesis chapters, so I use "I". Below I provide the details of the different collaborations. Unless otherwise stated, the work is my own.

Chapter 2
I performed the data analysis with guidance from Darren Obbard.

Chapter 3
The experiments were designed by me and Sarah Reece. The analysis of molecular data was performed by me with guidance from Darren Obbard.

Chapter 4
The experiments were designed by me and Sarah Reece. Honours students Suzanne Brierly and Lucy Carter assisted in data collection. Theoretical modeling was done by Andy Gardner and João Alpedrinha, using my biological knowledge.

Chapter 5
The experiments were designed during discussions between me, Sarah Reece, Nicole Mideo and Laura Pollitt. Honours students Anna ffrench-Constant, Debbie Mathers and Eve Hadshar assisted with data collection.

Some of the preliminary work for chapters 3 and 4 formed part of my undergraduate thesis, otherwise no work has not been submitted for any other degree or professional qualification

Ricardo Ramiro, 2nd July 2012

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I also have huge thanks to say to all of the Reece group (a.k.a. Reece's pieces). First, Laura... it's hard to find something not to thank you for! From the science chats to the pub, with some 'banana dances' in between, you were there at all moments! Nicole, for the great science chats and the beautifully silly pub chats! Will, for teaching me everything I know about animal work and for being a great source of (sometimes high class) entertainment! Aidan, for all the help in so many things that cannot be numbered, for the jokes in the pub and the sometimes awkward smiles they generate in others (as much part of the joke as the joke itself!). Vincent, for your help with ELISAs, and for the entertaining politics chats that made the long hours in the mouse house more enjoyable! Petra, for all your help in the lab and all your patience in replying my numerous questions... and for bringing us all together in numerous and amazing dinners! To Lucy and Ronnie for all your help in the lab.

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And last but very much not least, to Filipa Antunes... for all your encouragement and support, even when I clearly did not deserve it!

1 General Introduction

In the middle of last century, the end of 'the age of infectious diseases' was thought be imminent thanks to the development of drugs and vaccines (Fauci 2001). This forecast has been proved wrong for a variety of reasons, including the failure to appreciate the evolutionary potential of parasites (broadly defined as including virus, bacteria and eukaryotic micro- and macroparasites). For example, visits to hospital now risk the acquisition of antibiotic-resistant infections (Peleg and Hooper 2010), 'totally drug-resistant' tuberculosis has recently been identified in India (Udwadia et al. 2011), and resistance to the last frontline antimalarial drug is emerging (Cheeseman et al. 2012). Whilst drugs and vaccines clearly play an important role in the control of many infectious diseases, eradication has only been successful in a few cases (e.g. smallpox; Fenner 1982). Given that infectious diseases remain responsible for considerable morbidity and mortality of humans (e.g. in 2008 infectious diseases caused the death of 8.7 million people world wide; WHO 2008), wildlife, livestock, agricultural crops, it is surprising (and concerning) that evolutionary thinking is not better integrated into biomedicine and control policies.

Parasites are capable of fast evolution for a variety of reasons, including: short generation times, large population sizes, and considerable genetic and phenotypic variation can be generated through sexual reproduction, variable mutation rates, or the uptake and incorporation of external DNA. This makes understanding parasite evolution important from an applied perspective, but also makes parasites interesting and useful models for studying basic evolutionary processes (Poulin 2007; Stearns and Koella 2008). Yet in evolutionary biology and ecology, interest in parasite evolution has only taken off in the last few decades. The divide between the study of parasites and disease and the study of evolutionary and ecological processes has persisted partly due to the different approaches used by these fields. The biology of parasites has been traditionally studied by disciplines that ask proximate questions about the mechanisms of disease (parasitology, microbiology, cellular and molecular biology, immunology), whereas evolutionary biologists and ecologists avoid

mechanistic details in their search for general principles (Mideo et al. *in press*). However, progress is being made in the integration of evolutionary thinking into biomedicine. Evolutionary biologists have recently provided important and broad insights into the biology of pathogens, ranging from understanding parasite population structure (Mu et al. 2005), to the conditions that favor host shifts (Antonovics et al. 2002; Longdon et al. 2011), and the evolutionary impact of vaccination and drug treatment (Levin et al. 2000; Atkins et al. 2011). Furthermore, evolutionary tools are also starting to be used to directly inform policy decisions, for example in assessing the pandemic potential of new strains of influenza (Fraser et al. 2009).

Evolutionary biologists and ecologists are also offering novel insight into why parasites are the way they are. For example, there is a considerable body of work asking why parasites harm and sometimes kill their hosts, given they depend on host resources for transmission (i.e. fitness; see (Alizon et al. 2009) for a recent review). The leading explanation for this is that parasite fitness is maximized at intermediate values of virulence. Specifically, to transmit, parasites must replicate within-hosts but this causes damage to the host. Thus a trade-off arises: for the parasite to increase its transmission, it has to cause more damage to the host, but this increases the risk of host death, which prematurely curtails transmission. The 'trade-off hypothesis' has been developed to consider how parasite virulence and transmission and host recovery evolve in response to a variety of ecological factors (Anderson and May 1982; Ewald 1983; Alizon et al. 2009). These factors can be divided into processes that act at the within- and at the between-host levels. Factors that act at the between host level include: demography (e.g. density and spatial structure of susceptible hosts), the scale of transmission (e.g. local vs. global), the mode (e.g. horizontal vs. vertical) and route (e.g. vector, water) of transmission (Frank 1996; Lipsitch et al. 1996; Ebert and Mangin 1997; Boots and Mealor 2007; Poulin 2007). On the other hand, factors that act at the within host level include: immune responses, nutritional status of the host, co-infection (by con- or heterospecifics), host sex and age, and medical interventions (e.g. drugs and vaccines; Frank 1996; Beck et al. 2004; Mackinnon and Read 2004a; Knowles 2011; Read et al. 2011).

Within-host factors can vary considerably across hosts and during infections and there is increasing evidence that parasite traits have evolved to cope with variable environmental conditions (Poulin 2007). Consequently, there is increasing interest in explaining how ecological interactions (between parasites and between parasites and hosts) shape parasite traits, infection dynamics, and thus, scale up to affect transmission and epidemiology (May and Anderson 1983; Pedersen and Fenton 2007; Mideo et al. 2008; Restif 2009; Mideo and Reece 2012). Put simply, within-host processes determine the performance of parasites during infections, and between-host processes influence what kinds of host parasites subsequently infect. Advances in integrating within- and between-host processes have started to be made at the theoretical level (e.g. (Antia et al. 1994; Mideo et al. 2008; Day et al. 2011). However, experimental work is still lagging behind because parasite systems are required where there is a good understanding of within-host ecology (e.g. the nature of interactions between parasites and immune responses), parasites can be tracked through time during infections and in populations (i.e. across generations), the within- and the between-host environments can be perturbed (e.g. vary host immune status), and where parasite traits that are relevant for survival and transmission can be measured. Despite some shortcomings in the above areas, malaria (*Plasmodium*) parasites are one of the best candidate systems. Thanks to their applied relevance there are well-established rodent models for which a variety of molecular, cellular, genetic, immunological, and imaging tools exist (e.g. rodent malaria parasites can be genetically manipulated; the murine immune system and physiology is one of the most well studied among vertebrates), and the epidemiology of many populations is well documented (Carlton et al. 2001; Hay et al. 2002; Müller et al. 2003; Amino et al. 2005; Franke-Fayard et al. 2006; Stephens et al. 2012). The drawbacks of malaria model systems are that an entire life-cycle takes ~1 month (Killick-Kendrick and Peters 1978; Baton and Ranford-Cartwright 2005), manipulating factors that affect the between-host dynamics (e.g. host demography) can be very challenging, and the requirement of vector transmission complicates the integration of within- and between-host processes.

In the past 20 years, rodent malaria parasites have become an important model for studying the evolution and ecology of infectious disease, with a focus on how transmission and virulence evolve (Mackinnon and Read 2004b). However, the actual parasite traits that underpin virulence and transmission, especially those involved in the transition from host to vector have received much less attention (Mideo and Reece 2012). In this thesis I apply theory for life history evolution, speciation, sex allocation, and ecology to investigate how conditions experienced in the host and vector shape traits underpinning the virulence and transmission phenotypes of malaria parasites. To do this, I use tools from molecular and cellular biology, evolutionary genetics, theoretical biology, behavioural ecology, and immunology, which allow me to study processes on both evolutionary and ecological timescales. While the main aim is to obtain a greater understanding of the ecological factors influencing disease dynamics, my experiments also deal with concepts that apply to all dioecious organisms (e.g. reproductive success, resource availability, sex allocation) and therefore I provide insight into the generality of evolutionary and ecological theories. The work I present in this thesis has the following four components:

1. Phylogenetics: I provide an improved phylogenetic context for explaining and predicting the evolution of rodent malaria parasites. I use molecular and population genetics tools to reveal the relationships between strains, subspecies, and species, and identify how selection has shaped their genomes.

2. Reproductive isolation: By testing whether hybridisation can occur between different species of malaria parasite, I identify female mating ligands that are key to maintaining reproductive isolation. I also use molecular evolution methods to identify how selection has shaped the evolution of genes involved in fertilisation in malaria parasites.

3. Sex allocation: I experimentally test the assumption of sex allocation theory for malaria parasites that immune factors have sex-specific effects on the production and/or fertility of sexual stages and gametes. In collaboration,

I then develop a theoretical model to predict how the evolutionary trajectories of parasite sex ratio strategies are shaped by sex differences in gamete production, fertility and offspring development.

4. Infection dynamics in mixed-species infections: I use species with preferences for different ages of red blood cell to investigate the consequences of variation in resource availability and immunity for the dynamics of mixed-species infections. I experimentally manipulate resources, immunity, and competition in several different ways and study the performance of parasites in primary and secondary infections.

Due to the broad nature of the topics covered in the chapters, each chapter has its own introduction. Here, I describe the life cycle of malaria parasites before giving an overview of the theory behind the research topics I have investigated.

1.1 THE LIFE CYCLE OF MALARIA PARASITES

Malaria and related parasites are responsible for some of the most serious infectious diseases of humans, livestock, and wildlife. There are four species that specialise on human hosts (*P. falciparum, P. ovale, P. malariae, P. vixax*) with a 5th species (*P. knowlesi*) emerging in Southeast Asia (Singh et al. 2004). *P. falciparum* is considered to be the most virulent of the human parasites, but *P. vixax* is also responsible for considerable morbidity (Mendis et al. 2001). The four species of rodent malaria parasite (*P. berghei, P. chabaudi, P. yoelii, P. vinckei*) mirror many of the basic features of infections with human parasites and have a long history of success as model systems for malaria pathology, immunology, functional genetics, and pharmacokinetics (Killick-Kendrick and Peters 1978; Janse et al. 2006).

The *Plasmodium* life cycle (Figure 1.1) starts when an infected vector bites a host and releases infective stages (sporozoites; Amino et al. 2006). Sporozoites migrate through the bloodstream and invade the liver, starting the pre-erythrocytic phase (Prudêncio et al. 2006). Here, parasites will go through several rounds of replication and finally burst, releasing thousands of red blood cell (RBC) invading stages

(merozoites) into the blood. After invasion, merozoites grow and replicate inside the RBCs, producing progeny merozoites. RBCs then burst and the released merozoites find new RBCs to invade. This asexual cycle of RBC invasion-replication-bursting normally lasts for multiples of 24h and is responsible for many of the disease symptoms normally associated to malaria (e.g. anaemia, fever). Every cycle a small proportion (< 1%) of the asexually replicating parasites will develop into terminally differentiated male and female stages, termed gametocytes (Muirhead-Thomson 1954; Smalley et al. 1981; Smith et al. 2002). Gametocytes circulate in the blood and are responsible for transmission to the vector. Once a vector bites an infected host, gametocytes rapidly differentiate into gametes and male gametes locate and fertilize female gametes (Micks et al. 1948; Sinden 1983a; Sinden 1983b; Janse et al. 1986). Each male gametocyte differentiates into up to eight sperm-like gametes whereas each female gametocyte differentiates into a single spherical gamete (Sinden 1983a). The fertilized females (zygotes) then undergo a series of morphological transformations to form ookinetes. The ookinete is a motile and invasive stage that will cross the midgut wall within 18-20h of the mosquito blood meal (Sinden 1983a; Baton and Ranford-Cartwright 2005). After crossing the midgut wall, each ookinete forms an oocyst that produces thousands of sporozoites. Sporozoites then migrate to the salivary glands ready for transmission to a new host (Baton and Ranford-Cartwright 2005).

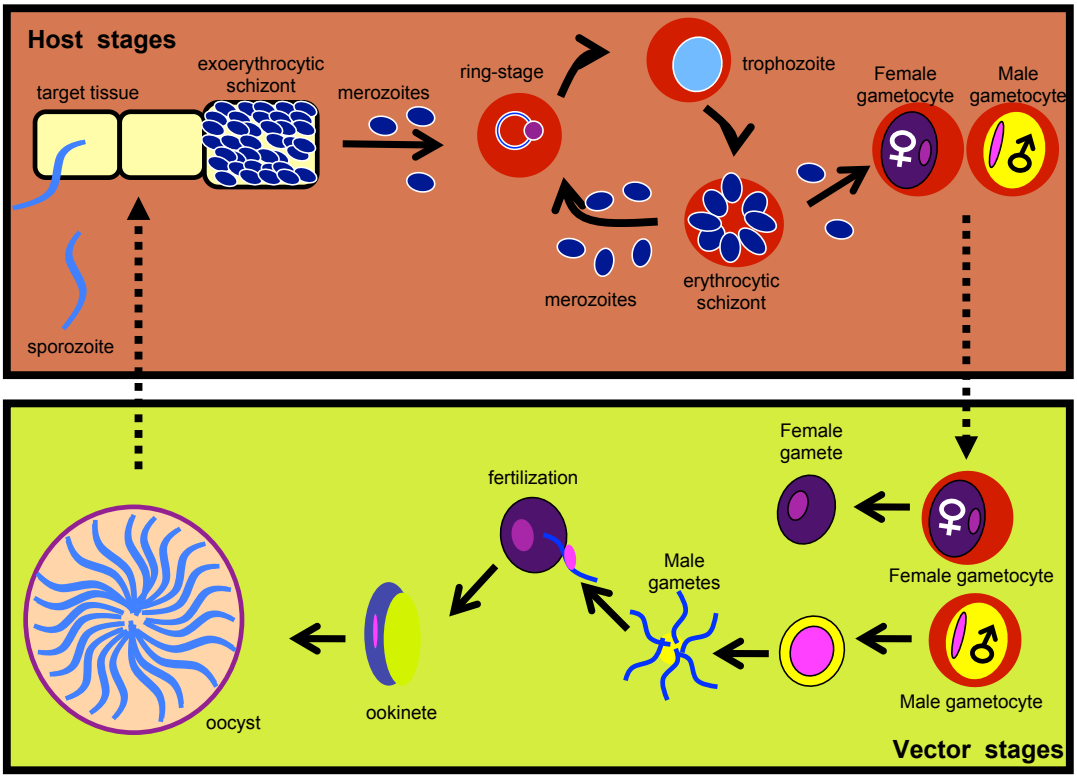

Figure 1.1 The life cycle of malaria parasites.

Transitions between different stages and between hosts are indicated with bold or dashed arrows, respectively.

Note that even though the main features of the life-cycle are conserved across species (e.g. sexual reproduction in the vector, multiple rounds of asexual replication in the host, etc), there is substantial variation between species in a variety of quantitative aspects (e.g. number of merozoites per schizont, densities of sexual stages), the development times and lifespans of asexual and sexual stages, and also in some qualitative factors (e.g. RBC age preference, the production of dormant stages; Killick-Kendrick and Peters 1978; Reece et al. 2009).

1.2 PHYLOGENETICS, REPRODUCTIVE ISOLATION, AND HYBRIDISATION

Evolutionary theory is based on the idea that all organisms are historically related through common ancestry. Modern molecular phylogenetic methods allow robust inference of these historical relationships across multiple levels of organization from genes to species (Whelan 2011). Phylogenetic trees for *Plasmodium* and its relatives

do exist but only one study has specifically addressed the phylogeny of rodent malaria parasites (Perkins et al. 2007). This analysis is consistent with earlier broad-scale taxonomic divisions (Killick-Kendrick and Peters 1978) but revealed inconsistencies in the level of genetic divergence between genotypes, subspecies and species (Perkins et al. 2007). Species level phylogenies are required to provide an evolutionary context for understanding multiple biological problems including ecological interactions, inferring divergence times, speciation patterns or inferring patterns of natural selection, etc (Whelan 2011). Species level phylogenies are also essential for predicting which lineages are reproductively isolated and so, enable cases of hybridisation and/or introgression to be identified (Whelan 2011). Whilst the number of *Plasmodium* species, their phylogeny, and divergence times have long been debated (Escalante and Ayala 1994; Ricklefs and Outlaw 2010; Sutherland et al. 2010; Prugnolle et al. 2011), the mechanisms leading to the origin and maintenance reproductive isolation have largely been ignored.

Reproductive isolation is essential for the maintenance of species identity and depends on the evolution of (normally, multiple) reproductive barriers (Coyne and Orr 2004). Reproductive barriers can be pre- and post-zygotic. Pre-zygotic barriers include habitat or temporal segregation, behavioural isolation (e.g. through mate choice), gametic incompatibility, lack of gamete transfer/activation (Coyne and Orr 2004; Barton et al. 2007). On the other hand post-zygotic barriers include hybrid sterility and inviability (at generations F_1 or F_2; Coyne and Orr 2004; Barton et al. 2007). While different reproductive barriers are relatively easy to identify, determining their individual contributions to reproductive isolation and identifying the mechanisms that led to their evolution is a substantially harder task (Coyne and Orr 1998; Coyne and Orr 2004; Barton et al. 2007; Matsubayashi and Katakura 2009; Nosil and Schluter 2011). Possible, non-mutually exclusive, mechanisms involved in the evolution of reproductive barriers include: genetic drift, divergent natural selection, sexual selection, and sexual conflict (Sobel et al. 2009). Despite the diversity of mechanisms that can lead to the evolution of reproductive isolation, five general patterns have been identified: (i) Natural selection will only act to directly increase reproductive isolation through pre-zygotic barriers. This is because avoiding

hybridisation normally brings a selective advantage to individuals, but once hybridisation has occurred, selection will not directly act to increase hybrid inviability or sterility (Barton et al. 2007); (ii) Post-zygotic barriers evolve gradually, leading to a 'speciation clock'. This may reflect the gradual accumulation of deleterious epistatic interactions between species (Coyne and Orr 1989, 1997, 1998; Sasa et al. 1998; Presgraves 2002; Price and Bouvier 2002); (iii) when one hybrid sex is unviable it is usually the heterogametic sex (Haldane's rule (Haldane 1922; Coyne and Orr 1998); (iv) pre-zygotic barriers often are a stronger barrier to gene flow than post-zygotic (Coyne and Orr 1998; Matsubayashi and Katakura 2009; Sánchez-Guillén et al. 2011); (v) gamete recognition proteins are often fast evolving and play an important role in reproductive isolation in organisms with external fertilisation (Palumbi 2009).

Contrary to reproductive isolation, hybridisation (when hybrids are viable) re-establishes gene flow between isolated species, potentially leading to homogenization of the gene pools of the hybridizing species. The frequency of hybridisation in nature has been widely debated, but recent developments in molecular tools have revealed hybridisation to be much more prevalent than previously thought (e.g. a survey showed that at least 10% of all animal species hybridize; Mallet 2005). This means that hybridisation can now be detected, but does it have an important role in evolution? Despite the fact that hybrids are usually unviable, two main lines of evidence suggest hybridisation is important. First, evidence suggesting that hybridisation or introgression (i.e. the gene flow from one species to another following hybridisation) can be adaptive is accumulating for a variety of organisms including sunflowers, mice, toads, and schistosomes (Rieseberg et al. 2003; Pfennig 2007; Huyse et al. 2009; Song et al. 2011). Second, contrary to conventional wisdom, hybridisation can be a facultative mate choice and may not just be due to a failure in species recognition (Veen et al. 2001; Pfennig 2007). For example, a recent study by (Pfennig 2007) shows that the fitness returns for female spadefoot toads of mating with con- or heterospecifics depends on environmental conditions and females adjust their mate preference adaptively, according to current environmental conditions.

While reproductive isolation and hybridisation have the potential to affect parasite evolution (Huyse et al. 2005; Detwiler and Criscione 2010), very little is known about how these processes influence parasites and scale up to affecting epidemiological dynamics, particularly for protozoan parasites. Since sexual reproduction is essential for disease transmission and mixed-species infections are common (Mayxay et al. 2004), determining whether, and how, different species are reproductively isolated is important. For example, if hybridisation occurs, introgression could result in drug resistance genes being exchanged between closely related species, even if hybrids have a low viability rate. Also, disrupting fertilisation is a key vaccine target (Carter 2001). As vaccination will tend to be species specific, this may change the interactions between species during mating, which could have unintended consequences.

1.3 SEX ALLOCATION

The study of sex allocation is grounded in a large body of theory that makes predictions about how organisms should adjust the sex ratio (proportion of males) they produce in response to a variety of factors, including: the inbreeding rate, cooperative and competitive interactions with kin, and maternal or paternal characteristics (West 2010). Because sex ratios are a relatively simple trait to model and empirically measure, theory has produced quantitative predictions that are often matched by data in a variety of experimental systems (from protozoan parasites to plants; West 2010). This has led sex allocation to be considered as 'the theory that best proves the power and accuracy of the Neodarwinian paradigm as a whole' (Hamilton 1996). As sex allocation is a large body of theory, I will introduce the relevant aspects for malaria parasites: local mate competition (LMC) and fertility insurance.

Local mate competition (LMC) predicts that, in subdivided populations, the sex ratio will depend on the inbreeding rate, such that the sex ratio will approximate 0.5 or 0 if the population is panmitic or fully inbred, respectively (Hamilton 1967; West 2010). A sex ratio of 0 is interpreted as the minimum number of males that must be

produced to fertilize all females (Hamilton 1967; West 2010). At high inbreeding rates (e.g. single genotype infections), female biased sex ratios are expected because this reduces competition between related males for access to females, and maximizes the number of females available to be fertilized (Hamilton 1967; Taylor 1981; Read et al. 1992b; West 2010). Specifically, because each male gametocyte makes several gametes, parasites should produce just enough males to provide gametes to fertilise all of the females (Read et al. 1992b). Producing more than the minimum number of males required reduces fitness returns because it is a waste of resources. On the other hand, when the inbreeding rate is low (e.g. infections with many conspecific strains), parasites should increase investment in males because this maximises the fitness returns from fertilizing females from different genotypes (Hamilton 1967; Read et al. 1992b; West 2010). For example in a single genotype infection, a very female biased sex ratio maximises the genotype's reproductive success, and when in a mixed-infection, a sex ratio closer to equality maximises the genotype's representation in the mating pool. In agreement with LMC theory, it has recently been shown that early in the infection, rodent malaria parasites adjust their sex ratio in relation to the inbreeding rate (Reece et al. 2008).

However, substantial variation in sex ratio patterns has also been shown throughout infections (when the inbreeding rate is constant; Paul et al. 2000; Reece et al. 2008; Mitri et al. 2009; Neal and Schall 2010), which cannot be explained by LMC. Moreover, data for related protozoan species fails to match the predictions of LMC (Shutler and Read 1998; West et al. 2000; West et al. 2001). Cases where sex ratios do not fit LMC may be explained by an extension termed 'fertility insurance' (West et al. 2002; Gardner et al. 2003; West 2010). Fertility insurance predicts that the optimal sex ratio should be less female biased than expected from the inbreeding rate if there is the risk that the number of males is not sufficient to fertilize all females (West et al. 2002; Gardner et al. 2003; West 2010). There are several reasons for why this could occur: (i) The number of male gametes produced per gametocyte could be a limiting factor (Read et al. 1992b; West et al. 2002; Gardner et al. 2003). Male gametocytes produce up to 8 male gametes, but data from rodent, human and lizard malaria show that they normally only achieve between 2 and 4 gametes,

suggesting that male gametes are very 'sperm' limited (Janse et al. 1986; Read et al. 1992a; Schall 2000; Reece et al. 2008). (ii) Low gametocyte density may lead to the stochastic risk that too few male gametocytes are taken up in the bloodmeal (West et al. 2002; Gardner et al. 2003). Low gametocyte densities could occur due to reduced investment in gametocytes or due to low red blood cell availability caused by anaemia. In agreement with the latter prediction, sex ratios have been observed to increase as anaemia develops (Reece et al. 2008). (iii) Gametocyte and/or gamete mortality may be sex specific and higher for males than females. Males are predicted to be more vulnerable than females to immune factors present in the bloodmeal, due to their more complex gametogenesis and mating activities (Carter et al. 1979; Rener et al. 1980; West et al. 2001; Paul et al. 2002; Gardner et al. 2003). Moreover, theoretical models show that the three factors above can interact synergistically, leading to an even less female biased sex ratio (and potentially to a male bias if the number of male gametes per gametocyte < 1) than predicted by LMC alone (Gardner et al. 2003). Fertility insurance is therefore predicted to place a lower bound on sex ratio.

While there is empirical support for the basic predictions and assumptions of LMC theory, testing the predictions and assumptions of fertility insurance theory is more challenging. Fertility insurance theory rests on the key assumption that the conditions that parasites encounter in the host negatively affect the fertility of males more than females when in the vector, and that parasites can correctly evaluate and respond to relevant in-host conditions (West et al. 2001; West et al. 2002; Gardner et al. 2003). Given the drive to develop transmission-blocking interventions that disrupt the fertility of one sex, it is particularly important to test these assumptions and determine how 'fine-tuned' parasite sex allocation actually is. For example, the efficacy of a vaccine that reduces the fertility of one sex may immediately be eroded if parasites facultatively respond by simply adjusting sex ratio in favor of the targeted sex.

1.4 INFECTION DYNAMICS IN MIXED-SPECIES INFECTIONS

Interspecies interactions are ubiquitous in nature and play a fundamental role in shaping ecological communities and in driving the evolution of organismal traits (Thompson 1999; Begon et al. 2006). Where organisms share a trophic level (e.g. primary consumers), interactions can span from being competitive, in which a focal species is negatively affected by interactions with another, to facilitation where a focal species benefits from the presence of another (Begon et al. 2006). Whilst competition has long been thought to play a major role in structuring ecological communities, the importance of facilitation has been overlooked (except in plants; Bruno et al. 2003). However, facilitation is an important process that has been shown to impact on individual fitness, population distributions, growth rates, and community structure (Bruno et al. 2003).

The type of ecological interactions (facilitation or competition) and their effects on the population dynamics of interacting species depend on the extent to which interactions are mediated by shared resources and/or shared enemies (e.g. predators; Holt 1977; Holt et al. 1994; Begon et al. 2006). Indeed, a common theme in ecology is the relative importance of 'top-down' (e.g. predators) or 'bottom-up' (e.g. resources) processes in determining species abundance and the nature of interspecies interactions (Hunter and Price 1992; Gurevitch et al. 2000; Begon et al. 2006). 'Exploitation competition' occurs when multiple species compete for access to a shared (and limited) pool of resources (Begon et al. 2006); 'apparent competition' occurs when an increase in the abundance of one species negatively impacts another by increasing the effect of a shared enemy (e.g. predator; Holt 1977; Begon et al. 2006). A third type of competition that may occur regardless of bottom-up or top-down processes is 'interference competition': when a species directly affects the growth/reproduction of its competitors (Begon et al. 2006). In contrast, facilitation can occur when one species increases the amount of resources available to another species or reduces interactions between natural enemies and another species (Bruno et al. 2003). All of these positive and negative interactions may affect all interacting species equally or have asymmetric effects.

While most research has focused on the ecological interactions of free-living communities, similar ecological rules should apply to parasite communities (Pedersen and Fenton 2007; Telfer et al. 2010). Research into host-parasite interactions has often studied one host-one parasite systems, but there is increasing recognition that mixed-species infections are the rule rather than the exception. Recently, Pedersen and Fenton (2007) proposed that within-host parasite communities can be represented as an interaction network with three trophic levels, in which the host immune response represents the top level, the co-infecting parasites represent the intermediate level, and host resources are the basal level. Such networks can link multiple parasites, branches of the immune response, and host resources, and therefore are useful for generating hypothesis about the mechanisms mediating interactions between species and for predicting outcome of those interactions. In accordance with this approach, Graham (2008) showed that in co-infections between helminths and microparasites, microparasite density is reduced if helminth species that induce anaemia are paired with microparasites that use RBCs as resources. On the other hand, microparasite density was increased if helminth infection led to the suppression of an inflammatory cytokine that is required for microparasite control. The mechanisms underpinning species interactions clearly have important impacts on infection dynamics, but they also matter because whether resources or immunity mediate species interactions can result in different evolutionary responses (for both hosts and parasites) to co-infection (Frank and Schmid-Hempel 2008; Mideo 2009; Choisy and De Roode 2010).

For malaria parasites, the majority of experimental work on mixed infections has focused on infections of conspecific genotypes. This research has produced overwhelming evidence that competition negatively affects all co-infecting genotypes (De Roode et al. 2004b; de Roode et al. 2005; Bell et al. 2006) through both exploitation (Antia et al. 2008) and immune-mediated apparent competition (Raberg et al. 2006). Moreover, the best competitors tend to be the more virulent genotypes. Several, non-exclusive, reasons may explain this: faster replication, the ability to infect a broader range of RBC ages, better immune evasion strategies, or 'safety in numbers' helps parasites survive immune responses (Antia et al. 2008;

Mideo et al. 2008; Mideo et al. 2011). However, there is reason to suspect that mixed-species infections pose different challenges to parasites and hosts and so virulence may not be favoured. For example, different parasite species are more immunologically distinct to each other than conspecifics and species have different RBC age preferences. This could reduce the impact of apparent competition and the effect of exploitation competition may vary according to RBC age preference. However, few experiments have manipulated both resources and immunity to examine how top-down and bottom-up factors affect interactions between co-infecting parasites (both con- and heterospecifics).

2. Molecular Evolution and Phylogenetics of Rodent Malaria Parasites

This chapter is in review at BMC Evolutionary Biology: Ramiro, RS, Reece, SE & Obbard, DJ. Molecular Evolution and Phylogenetics of Rodent Malaria Parasites.

2.1. SUMMARY

Over the last 6 decades, rodent *Plasmodium* species have become key model systems for understanding the basic biology of malaria parasites. Cell and molecular parasitology have made much progress in identifying genes underpinning interactions between malaria parasites, hosts, and vectors. However, little attention has been paid to the evolutionary genetics of parasites, which provides context for identifying potential therapeutic targets and for understanding the selective forces shaping parasites in natural populations. Additionally, understanding the relationships between species, subspecies, and strains, is necessary to maximise the utility of rodent malaria parasites as medically important infectious disease models, and for investigating the evolution of host-parasite interactions. We collected multi-locus sequence data from 58 rodent malaria genotypes distributed throughout 13 subspecies belonging to *P. berghei, P. chabaudi, P. vinckei,* and *P. yoelii*. We employ multi-locus methods to infer the subspecies phylogeny, and use population-genetic approaches to elucidate the selective patterns shaping the evolution of these organisms. Our results reveal a time-line for the evolution of rodent *Plasmodium* and suggest that all the subspecies are independently evolving lineages (i.e. species). We show that estimates of species-level polymorphism are inflated if subspecies are not explicitly recognized, and demonstrate that most loci are under purifying selection.

2.2. INTRODUCTION

Since their discovery in Central West Africa in the 1940s, rodent malaria parasites - *Plasmodium berghei*, *P. chabaudi*, *P. vinckei* and *P. yoelii* – have become the *de facto* animal models for the study of *Plasmodium* biology (Killick-Kendrick and Peters 1978). These parasites have proven useful to investigate diverse aspects of host-parasite-vector interactions, evaluate potential interventions for malaria control, and to generate and test hypothesis about the biology of human malaria (Killick-Kendrick and Peters 1978; Carlton et al. 2001; Stephens et al. 2012). Although, no single species of rodent *Plasmodium* is the perfect model for human parasites, different species have proven useful for different aspects of biology (Craig et al. 2012). For example, while *P. chabaudi* is a good model for the study of infection dynamics and immune responses (Stephens et al. 2012), *P. berghei* is a better model for studying the biology of transmission (Khan et al. 2005; Guttery et al. 2012). Nevertheless, while the rodent malarias are well studied in the lab, much less is known about their natural history and evolutionary genetics. This is in sharp contrast to human pathogens, where evolutionary genetics plays an important role, particularly in explaining the evolution of drug resistance and patterns of natural selection at target antigens (Conway 2007).

Although only a handful of genotypes are regularly used in the lab, the World Health Organization (WHO) Registry of Standard Malaria Parasites (in the European Malaria Reagent Repository, University of Edinburgh) contains over 70 field isolates of rodent malaria parasites. The availability of multiple isolates means that multi-locus sequence data and modern phylogenetic methods can be used to improve understanding of many aspects of the natural history of rodent malaria parasites. This includes identifying lineages that are independently evolving (species delimitation), estimating the 'species tree' (phylogeny), divergence times, and population sizes (Whelan 2011). Additionally, an improved phylogeny enables the better application of molecular evolution methods, as these often depend on an understanding of the species tree (e.g. (McDonald and Kreitman 1991).

Despite the variety of papers on *Plasmodium* phylogeny, only one has addressed the phylogeny of rodent malaria parasites (e.g. Escalante and Ayala 1994; Hayakawa et al. 2008; Pacheco et al. 2011). Perkins *et al*. (2007) sequenced fragments of 7 loci from the nuclear, mitochondrial and apicoplast genomes for 19 parasite genotypes covering all the species and 12 subspecies of rodent *Plasmodium*. Their results were consistent with the original species descriptions based on morphological and isozyme characteristics, but revealed inconsistencies in the level of genetic divergence between genotypes, subspecies and species (e.g. pairwise divergence between genotypes of different *P. vinckei* subspecies was shown to be much higher than between genotypes of *P. berghei* and *P. yoelii*(Killick-Kendrick and Peters 1978; Perkins et al. 2007). Here, we collect the largest rodent malaria sequence dataset to date, to resolve these inconsistencies, improve the phylogeny and generate a resource for future population genetic and experimental studies in these important model species.

We revived 58 rodent *Plasmodium* genotypes (some for the first time in 44 years), from 13 subspecies across four species and sequenced 11 'house-keeping' nuclear loci. We use these data to understand species delimitation and generate a multi-locus phylogeny. We infer the relative effective population sizes, generate hypotheses for the divergence times and describe the patterns of selection and constraint in these loci. First, our results suggest all rodent malaria subspecies are sufficiently genetically isolated that they could be considered species. Second, we estimate the (sub)species tree, divergence times and population sizes, under three different time-calibrations. The three alternative calibrations we used reflect current uncertainty in *Plasmodium* evolution and, as expected, resulted in very different divergence times that we discuss in relation to their assumptions. Finally, we estimate levels of linkage disequilibrium and polymorphism, and calculate summary statistics that reflect the form and strength of natural selection (K_A/K_S, McDonald-Kreitman tests and Tajima's D). Our results show that polymorphism and linkage disequilibrium are low and reveal that most loci are under purifying selection.

2.3. MATERIALS AND METHODS

2.3.1. Parasites and hosts

Rodent malaria parasites were isolated from the wild between 1948 and 1974, from 5 different countries (Cameroon, Central African Republic, Congo, Democratic Republic of the Congo and Nigeria; Killick-Kendrick and Peters 1978). During this period, four species were described, encompassing 13 subspecies. The 'thicket rats' *Thamnomys rutilans* (also known as *Grammomys poensis*) and *Grammomys surdaster* (also known as *Grammomys dolichurus*; Wilson and Reeder ; Killick-Kendrick and Peters 1978) were found to be the most common vertebrate hosts and whilst *Anopheles dureni millecampsi* was frequently infected by rodent malaria parasites, the vector for some of these parasite species remains unknown (reviewed in (Killick-Kendrick and Peters 1978); Table S2.1).

We revived 58 rodent malaria genotypes, spanning all known species and subspecies, from the WHO Registry of Standard Malaria Parasites (European Malaria Reagent Repository, University of Edinburgh; Table S2.1). Specifically, we obtained genotypes for: *P. berghei* (8 genotypes), *P. chabaudi adami* (2), *P. c. chabaudi* (14), *P. c.* subsp. (2), *P. vinckei brucechwatti* (2), *P. v. lentum* (3), *P. v. petteri* (3), *P. v.* vinckei. (2), *P. v.* subsp. (5), *P. yoelii killicki* (2), *P. y. nigeriensis* (2), *P. y. yoelii* (10) and *P. y.* subsp. (3). *P. c.* subsp., *P. v.* subsp. and *P. y.* subsp. refer to previously recognised, but as yet unnamed subspecies. To avoid genotypes previously subjected to artificial selective pressures (intentionally or unintentionally) and minimise host passages, we used parasite genotypes from stabilates frozen as close as possible to the date of collection/arrival in Edinburgh (dates in Table S2.1). Cryopreserved parasite stabilates (-80ºC) were defrosted and injected into MF1 male mice (in house supplier, University of Edinburgh) in 100µl carrier solution (Bell et al. 2006). When sufficient parasitaemia was visible in giemsa stained thin blood smears, blood samples were taken and DNA extracted (following Bell et al. 2006) for PCR and sequencing.

2.3.2. PCR and sequencing

We selected 11 nuclear genes, covering 3 chromosomes, mainly coding for 'house-keeping' functions (Table 2.1) and designed PCR primers (Table S2.2) using genome sequences available in PlasmoDB (http://plasmodb.org) for *P. berghei*, *P. chabaudi* and *P. yoelii*. We treated PCR products with Exonuclease 1 (New England Biolabs, UK) and Shrimp Alkaline Phosphatase (Sigma-Aldrich, UK) to remove unused PCR primers and dNTPs, and then sequenced in both directions using BigDye reagents (Applied Biosystems, UK) on an ABI 3730 capillary sequencer (Gene Pool Sequencing Facility, Edinburgh). For most genes, more than 85% of genotypes provided sequence data (Table 2.1).

2.3.3. Sequence analysis

We assembled sequences with Seqman (DNASTAR), inspected all polymorphisms manually and aligned sequences with ClustalW (in BioEdit; Hall 1999), with adjustments by eye. *Gdpgmp* was divided into intronic and exonic regions before analysis. We used GARD (Kosakovsky Pond et al. 2006b, a) in Datamonkey (Delport et al. 2010; Kosakovsky Pond et al. 2010) to test for recombination at each locus, but there was no statistical support for recombination in any loci. To provide time-calibrations, we incorporated publically available data for *cytochrome b* (*cytb*) and *cytochrome oxidase I* (*coI*; accession no. *coI*: DQ414589–DQ414606; *cytb*: AY099050, AY099051, DQ414545– DQ414660; Perkins et al. 2007).

We generated preliminary gene trees with BEAST (v1.6.2; Drummond 2007), which placed two genotypes outside of the expected taxon: (i) *P. v. vinckei* v-52 groups with *P. c. chabaudi*; and (ii) *P. v. vinckei* v-67 groups with *P. y. nigeriensis*. This was consistent across all 11 genes, indicating past misidentification and/or labelling error, and these sequences were retained under the new species identification suggested by gene trees. Interestingly, previous researchers had labelled *P. v. vinckei* v-67 as '*P.*

berghei-like' (Bafort 1971); on the basis of morphology), which is similar to *P. y. nigeriensis*.

Table 2.1 House-keeping loci selected for analysis.
Gene ID and predicted function were obtained from PlasmoDB. Maximum sequence length (length); Chromosome (chr).

gene	chr	gene ID	predicted function	length (bp)	no. of sequenced genotypes
26s	3	PBANKA_030360	proteasome 26S regulatory subunit	742	55
atpase	13	PBANKA_136260	nucleolar preribosomal associated cytoplasmic ATPase	716	53
cons	3	PBANKA_030790	conserved *Plasmodium* protein, unknown function	756	22
cyspro	13	PBANKA_132170	cysteine proteinase	811	52
dhfr	7	PBANKA_071930	bifunctional dihydrofolate reductase-thymidylate synthase	610	56
exonuc	3	PBANKA_030260	3'-5' exonuclease	591	47
gdpgmp	3	PBANKA_030880	GDP-fructose:GMP antiporter	256	50
glurna	13	PBANKA_136200	glutamate - tRNA ligase	563	40
hsp70	13	PBANKA_135720	heath shock protein 70	658	51
metrans	3	PBANKA_030390	metabolite/drug transporter	685	44
rnabind	13	PBANKA_135690	RNA-binding protein	554	56

2.3.4. Bayesian species delimitation

We performed a species delimitation analysis using the program Bayesian Phylogenetics and Phylogeography v2.0 (BPP; Rannala and Yang 2003). This is a coalescent-based method that uses the concordance of gene trees across multiple loci as evidence for a particular species delimitation model, but does not rely on reciprocal monophyly for each individual locus. It uses a reversible-jump Markov chain Monte Carlo (rjMCMC) algorithm to calculate the posterior distributions of different species delimitation models. The method is based on the biological species concept, assuming no migration following speciation and allowing for stochastic fluctuations in the coalescent process and lineage sorting due to ancestral polymorphism (Leache and Fujita 2010; Yang and Rannala 2010; Zhang et al. 2011).

BPP requires a user-specified guide tree as input to constrain the phylogeny and species delimitation space. The guide tree is a fully resolved tree, representing the most subdivided species delimitation model that is biologically plausible. The rjMCMC algorithm then evaluates the posterior probabilities of speciation models created by collapsing or splitting nodes on the guide tree (Yang and Rannala 2010). Rodent malaria subspecies have been previously defined on the basis of morphological characters and isozyme polymorphism (Killick-Kendrick and Peters 1978), thus we consider all the subspecies as operational taxonomic units and construct the guide tree with *BEAST (see *BEAST section below).

BPP uses gamma priors G(α,β) on the population size parameters (θ) and on the age of the root in the species tree (τ_0), with prior mean α/β and prior variance α/β^2. The other divergence time parameters were assigned the Dirichlet prior (equation 2 in (Yang and Rannala 2010). To confirm our results, we set up alternative models that tested for effects of: (i) algorithm and fine-tuning parameters; (ii) θ prior; and (iii) number of loci (Tables S2.3-4). The τ_0 prior was G(1.5,30) for all analyses and was selected based on

estimates from (Hayakawa et al. 2008; Ricklefs and Outlaw 2010). We used $\alpha = 1.5$ for all priors to make them diffuse, given that we have little information on their true values. We ran each analysis in duplicate, for $5x10^6$ steps (sampling every 50), with a burn-in of $5x10^4$ and confirmed that the starting speciation model was different for replicate runs (this is important to test for the stability of the results).

2.3.5. Genealogical sorting index (gsi)

We validated results from BPP using the genealogical sorting index, which quantifies exclusive ancestry on a scale of 0 to 1, where 1 represents complete monophyly (Cummings et al. 2008). We calculated both *gsi* (single genes) and gsi_T (multiple genes), using the genealogical sorting index web interface (http://www.genealogicalsorting.org/), for the entire dataset and for a reduced dataset (5 loci). The input gene trees were maximum clade credibility gene trees generated in BEAST, as referred in Sequence Analysis. The significance of *gsi* and gsi_T is evaluated by comparing the values obtained with the null hypothesis that the amount of exclusive ancestry observed is the same as might have been observed at random. This was done by generating 10000 permutations on the subspecies labels, while holding the tree constant, and computing *gsi* for each permutation. A *p-value* was then computed as the probability of randomly obtaining *gsi* values that are equal to or greater than the observed *gsi* value (Cummings et al. 2008). As our dataset is unbalanced and it is known that uneven sample sizes can lead to an underestimation of the *p-values* for small group sizes, we assessed significance at 0.001 (Cummings et al. 2008; Polihronakis 2009; Gazis et al. 2011; Niemiller et al. 2012).

2.3.6. *BEAST: BPP guide tree, species tree, divergence times and population sizes

*BEAST from the software package BEAST (Drummond 2007; Heled and Drummond 2010) is an MCMC method that estimates the species tree directly from multi-locus sequence data, under the multispecies coalescent model. It assumes that discrepancies

between gene trees are due to incomplete lineage sorting rather than gene flow and incorporates uncertainty in nucleotide substitution model parameters and the coalescent process. We used *BEAST to estimate the guide tree for BPP, to infer the species tree and to estimate divergence times and population sizes.

2.3.6.1. BPP guide tree

To generate the guide tree, we performed two independent runs, for $1.1x10^9$ states (sampling every 10^5 states and excluding the first 10% as burn-in) using the entire nuclear dataset and the mitochondrial loci. We used the HKY (Hasegawa et al. 1985) substitution model with the dataset partitioned into 3 codon positions, no site heterogeneity, substitution models for all nuclear loci linked (apart from the *gdpgmp* intron) and a strict clock. We applied a Yule process speciation prior for species branching rates and a piecewise linear and constant root model for population sizes. We assessed convergence by evaluating the sampled values, for each parameter, across time in Tracer v1.5 (http://beast.bio.ed.ac.uk/Tracer). As effective sample sizes (ESS) were above 200 and replicate runs converged, we combined tree and log files with LogCombiner v1.6.2 (http://beast.bio.ed.ac.uk).

2.3.6.2. Species tree inference, divergence times and population sizes

Using the preferred speciation model from BPP (i.e. fully-resolved subspecies), we estimated the species tree together with divergence times and population sizes for three calibration methods. We set up replicate runs of each file, assessed convergence, and combined log and tree files as above.

2.3.6.3. Calibration methods

A variety of approaches have been used to estimate divergence times in malaria parasites, and as yet there seems to be little consensus on the age of key divergences (Escalante and Ayala 1994; Hayakawa et al. 2008; Ricklefs and Outlaw 2010; Pacheco

et al. 2011). To reflect this uncertainty, we selected three calibrations as alternative priors for dates of common ancestry in rodent *Plasmodium* (Ricklefs and Outlaw 2010; Pacheco et al. 2011). For simplicity, we denote the three alternative calibrations as Ricklefs2010, Pacheco2011-A and Pacheco2011-B, corresponding to the first author and year in which the estimates were published. The Ricklefs2010 calibration was obtained from (Ricklefs and Outlaw 2010), in which the authors used *cytb* data to estimate the ratio of parasite to host substitution rates from endemic parasites and birds from the West Indies. This ratio was then multiplied by an estimate of the host's *cytb* nucleotide divergence rate, yielding an estimate for the *Plasmodium* molecular clock with mean 0.012 genetic divergence per million years and standard deviation 0.002 (Ricklefs and Outlaw 2010). We implemented this as a normal prior on the *cytb* clock rate. Both Pacheco2011 calibrations are estimates for the root height of rodent malaria parasites. These were obtained from (Pacheco et al. 2011), in which a tree of malaria parasites from mammals, birds and reptiles was calibrated at the *Papio*/*Macaca* divergence (i.e. assuming host-parasite co-divergence; Pacheco2011-A) and the latter plus the Human/*Macaca* divergence (Pacheco2011-B), suggesting a most recent common ancestor for the mitochondrial DNA of all rodent malaria at 8.32 Mya (95% Highest Posterior Density interval [HPD]: 5.10-12.60) and 14.2 Mya (9.96-18.89), respectively (see Table 2.3 in (Pacheco et al. 2011). From these estimates, we derived gamma-distributed priors for the root of *coI* and *cytb* (Figures S2.1-2). Importantly, these estimates are consistent with the occurrence of malaria in lemurs in Madagascar, which could not have been acquired after the last terrestrial mammal colonization event (~20 million years ago [Mya]; Poux et al. 2005; Ali and Huber 2010; Pacheco et al. 2011).

2.3.7. Linkage disequilibrium

To estimate linkage disequilibrium (LD), we created a concatenated alignment of the 11 nuclear genes and used the R packages popgen and LDheatmap (R v2.14.0) to calculate and visualize r^2 for pairs of single nucleotide polymorphisms (SNPs; Shin et al. 2006; Marchini 2011; R Development Team Core 2011). Because we observe almost no

polymorphism in *P. berghei*, and the number of genotypes in each of the *P. vinckei* subspecies is low, we only analyse *P. chabaudi* and *P. yoelii* and their subspecies *P. c. chabaudi* and *P. y. yoelii*.

2.3.8. Molecular evolution

We used DNAsp v5 (Librado and Rozas 2009) to compute the following statistics for each gene: McDonald-Kreitman (MK) tests (α: proportion of non-synonymous substitutions attributable to positive selection; McDonald and Kreitman 1991); Tajima's *D* at silent sites (Tajima 1989), polymorphism (π_A, π_S and π_A/π_S) and divergence (K_A, K_S and K_A/K_S; Nei and Gojobori 1986). To infer patterns of substitution independently for each lineage, we measured divergence from a putative ancestral sequence inferred by maximum likelihood for the *P. berghei-P. yoelii* and *P. chabaudi-P. vinckei* nodes, under a codon model using PAML v4.5 (Yang 2007). In this analysis we used either the species or the subspecies with the most genotypes (*P. c. chabaudi*, *P. v.* subsp. and *P. y. yoelii*) as the intraspecific groups. We also performed this analysis for *P. v. petteri* because this subspecies was isolated from the same location as *P. c. chabaudi* and *P. y. yoelii*. To test for significant differences between taxa in π_A, π_S, π_A/π_S, K_A, K_S and K_A/K_S we used pair wise Wilcoxon rank sum tests with *p-values* adjusted by Bonferroni correction in R (R Development Team Core 2011).

Finally, we used a maximum likelihood version of the MK test (Welch 2006) to test for variation in α between loci and/or lineages and to estimate mean α from multi-locus data. This software implements several likelihood models, which can be used to test different hypotheses about selection at loci of interest. We set up three models, in which: (i) α is constrained to zero at all loci, i.e. no adaptive evolution; (ii) α is a free-parameter common to all loci; and (iii) α can take a different value at each locus. Due to small sample sizes, we compared models using AICc and calculated Akaike weights following (Burnham and Anderson 2002) Akaike weights represent the probability that a model is true, given that the true model is amongst those tested). To estimate $\bar{\alpha}$ and 95%

confidence intervals across loci, we performed 1000 bootstraps on the results from model *ii*. We used the resulting distributions to test whether $\bar{\alpha}$ significantly differed between lineages (i.e. to detect lineage-specific selection patterns). We performed this analysis for the subspecies *P. c. chabaudi*, *P. v.* subsp. and *P. y. yoelii*, using polymorphism and divergence counts obtained as described above (see Table S2.8). Compared to other loci, *cyspro* in *P. c. chabaudi* and *P. v.* subsp. shows extremely high between-species divergence. This may reflect a substantial underlying change in its biology, and we therefore performed the analysis both including and excluding this locus.

2.4. RESULTS

2.4.1. Species delimitation: BPP and gsi

BPP analyses overwhelmingly supported the fully-resolved guide tree (Figure 2.1) with posterior support always exceeding 0.86, and very close to 1 for the 11 gene dataset (Tables S2.3-4). In all analyses, the secondary sampled models consistently collapsed nodes *e*, *h*, *and l* in Figure 2.1. The consistency of our results across replicate runs and with different algorithms (and fine-tune parameters) indicates that mixing of the BPP rjMCMC algorithms was good. To evaluate the validity of these results we used the genealogical sorting index (*gsi*) as an independent statistic (Niemiller et al. 2012). We present only the gsi_T values for the delimited species, in Table S2.5. The great majority of gsi_T are above 0.7, with all but two (*P. y. killicki* and *P. y.* subsp.) being significant. This suggests a high level of exclusive ancestry for the majority of the subspecies. We obtained similar results, for both BPP and *gsi*, with a reduced dataset. Thus, the results from BPP and *gsi* are robust and in agreement, suggesting that all 13 of the rodent malaria subspecies are independently evolving lineages.

2.4.2. Species tree inference, divergence times and population sizes

As the fully-resolved subspecies tree was preferred by BPP, we used the different subspecies as the operational taxonomic units for estimating the time-calibrated multi-locus species tree in *BEAST. We evaluated mixing of the MCMC in all runs and observed that the ESS was always above 200, with replicate runs converging towards the same parameter values.

As expected, in our species tree, *P. chabaudi* and *P. vinckei* form a clade, *P. berghei* and *P. yoelii* form another clade, and all the subspecies nest within their corresponding species (Figure 2.1 and S2.1-S2.2). Table 2.2 shows the inferred divergence times depending on the different calibrations used. The Ricklefs2010 calibration resulted in estimates that fall within the Pacheco2011-A Highest Posterior Densities (HPDs). Thus, we only present estimates for the Pacheco2011 calibrations. As expected, the Pacheco2011-A resulted in younger estimates than Pacheco2011-B, with point estimates for the major splits between *P. berghei – P. yoelii* and between *P. chabaudi – P. vinckei* ranging from 1-4 and 2.5-9 Mya, respectively. From all analyses, it is clear that *P. chabaudi* and *P. vinckei* diverged much earlier than *P. berghei* and *P. yoelii*, with the latter divergence occurring simultaneously or after the divergence of the subspecies *P. v. brucechwatti*, *P. v. lentum* and *P. v. vinckei*.

In general, the effective population sizes under the *BEAST model appeared to mix well. However, for the end population sizes, ESS were often below 200 (particularly for the Pacheco2011-B calibration). Thus, we treat these results with caution and discuss the population sizes only from a qualitative perspective for the tips of the tree (i.e. the subspecies) and not for the ancestral nodes. As expected, relative *BEAST estimates of effective population sizes generally reflect estimates of neutral genetic diversity (π_S), and suggest that *P. berghei* and *P. v. vinckei* have the smallest population sizes, whereas *P. c. chabaudi*, *P. c. adami* and *P. y. yoelii* have the largest (Table S2.6).

2.4.3. Linkage Disequilibrium

Within subspecies (*P. c. chabaudi* and *P. y. yoelii*; Figure S2.3) we were unable to detect substantial LD between any pairs of sites, suggesting either that LD is extremely low, or that the small sample sizes for individual subspecies results in low power. However, when we calculated LD by grouping all subspecies within their species (*P. chabaudi* and *P. yoelii*; Figure S2.4), substantial LD could be detected within but not between genes.

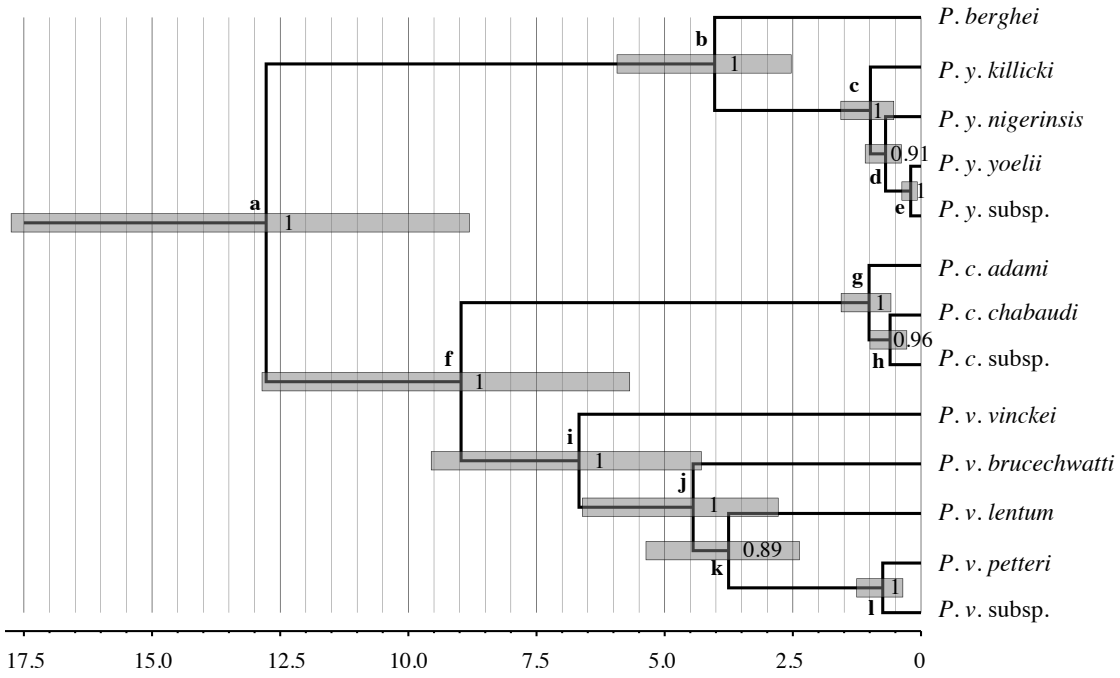

Figure 2.1 Rodent malaria phylogeny and divergence times for the Pacheco2011-B calibration.

This *BEAST time-tree is an example of the divergence times we obtained, though the results from Pacheco2011-A should also be taken into account (see Table 2.2; Figure S2.1). Node bars represent 95% HPD and axis is in Mya. Internal node labels represent posterior support and external node labels correspond to the divergence times in Table 2.2. The topology of this tree was the same as the guide tree for BPP.

Table 2.2 Divergence times as estimated by *BEAST.

The combined 95% HPD for the Pacheco2011 calibrations represent our best divergence time estimates. Nodes are labelled as in Figure 2.1. Values in brackets represent 95% HPD.

node	Pacheco2011-A (Mya)	Pacheco2011-B (Mya)	Pacheco2011 combined 95% HPD (Mya)
a*	7.3 (4.5; 11.1)	13.1 (9.0; 17.9)	4.5 – 17.9
b	2.3 (1.2; 3.6)	4.1 (2.5; 5.9)	1.2 – 5.9
c	0.6 (0.3; 0.9)	1.0 (0.5; 1.6)	0.3 – 1.6
d	0.4 (0.2; 0.7)	0.7 (0.4; 1.1)	0.2 – 1.1
e	0.1 (0.03; 0.2)	0.2 (0.07; 0.4)	0.03 – 0.4
f	5.1 (2.9; 8.0)	9.2 (5.7; 12.9)	2.9 – 12.9
g	0.6 (0.3; 0.9)	1.0 (0.6; 1.6)	0.3 – 1.6
h	0.4 (0.1; 0.6)	0.6 (0.3; 1.0)	0.1 – 1.0
i	3.8 (2.1; 5.9)	6.8 (4.3; 9.6)	2.1 – 9.6
j	2.6 (1.4; 4.0)	4.6 (2.8; 6.6)	1.4 – 6.6
k	2.2 (1.2; 3.4)	3.9 (2.5; 5.5)	1.2 – 5.5
l	0.4 (0.2; 0.8)	0.8 (0.5; 1.3)	0.2 – 1.3

*our posterior for the basal split of rodent *Plasmodium* (node *a*) differs slightly from our priors. This is because we set up calibrations on the root height of the linked mitochondrial gene tree, rather than on the root height of the species tree (i.e. including all loci).

2.4.4. Molecular evolution

We obtained estimates of polymorphism (π_A, π_S and π_A/π_S) and divergence (K_A, K_S and K_A/K_S), α (MK tests) and Tajima's D at silent sites for each gene in our analysis (Tables S2.7-8). Variation in genotype coverage (Table 2.1) and an absence of polymorphism

and/or divergence in some taxa meant we could not obtain these statistics for every gene in all lineages. For both α and Tajima's D at silent sites, there was only one result significantly incompatible with a standard neutral model: *cyspro* in *P. chabaudi* (p = 0.016) and *hsp70* in *P. yoelii* ($p < 0.05$), respectively. However, these are unlikely to remain significant after correction for multiple tests and should be treated with caution. The distribution of divergence (Figure 2.2) is similar across lineages with no significant differences between them. Although there were some neutrally evolving lineage-specific exceptions, K_A/K_S is generally low (~0.2), suggesting that these genes are under purifying selection across most parasite lineages. On the other hand, polymorphism varies between lineages and significant differences could be detected, particularly for *P. vinckei* (Figure 2.2; Table S2.9). As other researchers have shown (Saula et al. 1997; Perkins et al. 2007), polymorphism in *P. berghei* was extremely low and we only detected two non-synonymous SNPs in *cyspro* for the K173 and K173N genotypes (Table S2.7).

Finally, we used a maximum likelihood MK test to analyse whether α varies between loci and/or lineages. We show that variation in α between loci is restricted to *P. c. chabaudi* and *P. v.* subsp. and is influenced by the presence of *cyspro*. In the absence of *cyspro*, the best models always include α constrained to zero (model *i*) or as a free-parameter common to all loci (model *ii*), with model *i* having substantially higher Akaike weights for *P. v.* subsp. and *P. y. yoelii*, indicating there is little support for positive selection in these lineages. Additionally, the 95% bootstrap intervals for α were wide, and α did not vary significantly between lineages

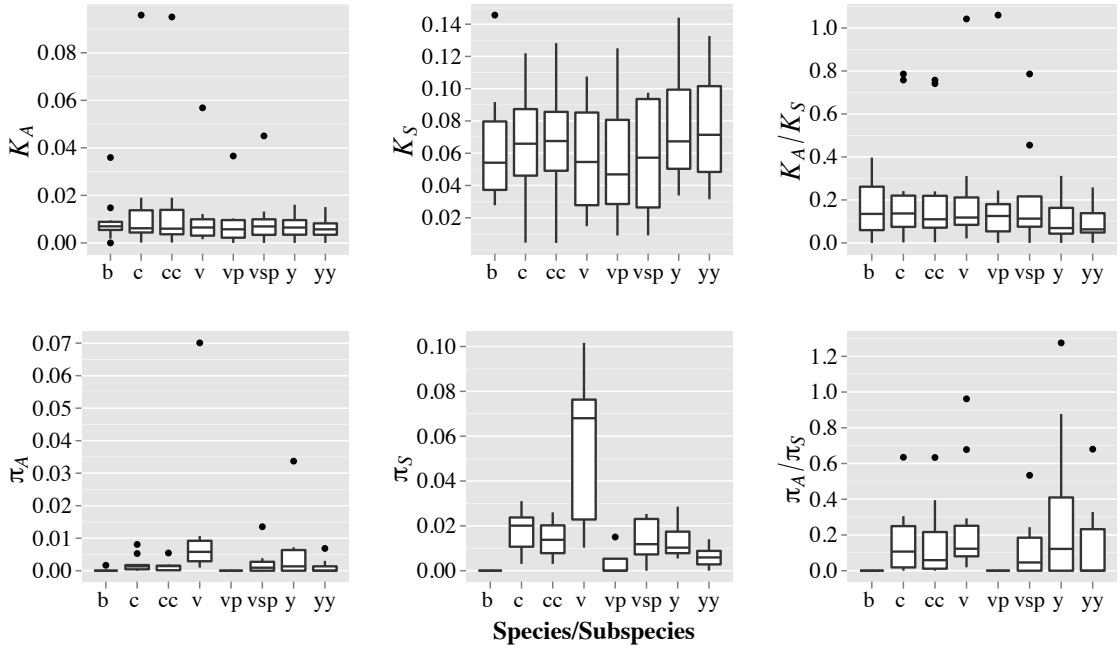

Figure 2.2 Divergence and polymorphism in rodent malaria parasites.

Boxplots were constructed using values obtained for each of the 11 nuclear loci (Tables S2.7-8) and represent divergence (top row) and polymorphism (bottom) at non-synonymoys sites (left; K_A, π_A), synonymous sites (middle; K_S, π_S) and their ratio (right; K_A/K_S, π_A/π_S). Estimates were obtained for: *P. berghei* (b); *P. chabaudi* (c); *P. c. chabaudi* (cc); *P. vinckei* (v); *P. v. petteri* (vp); *P. v.* subsp. (vsp); *P. yoelii* (y); *P. y. yoelii* (yy).

2.5. Discussion

By sequencing multiple loci from 58 genotypes across four species of rodent *Plasmodium*, we clarify the evolutionary context to study both the population genetics and functional biology of this group. First, our results suggest that all subspecies are independently evolving lineages and that the number of rodent malaria species may be underestimated. Second, we suggest two different timelines for the evolution of rodent malaria parasites (with point estimates for the *P. berghei – P. yoelii* and the *P. chabaudi – P. vinckei* splits ranging from 1-3 and 3-13 Mya, respectively). Third, we find that

most of the 11 nuclear loci used are under purifying selection, although there is some evidence that a potential vaccine candidate (*cyspro*) may be under positive selection. In agreement with other studies, we show that polymorphism is almost absent (but non-zero) in isolates of *P. berghei*. Finally, we find that linkage disequilibrium is generally low in *P. yoelii* and *P. chabaudi*.

All analyses suggest that the rodent malaria subspecies could be considered species (with the possible exception of the unnamed *P. y.* subsp.; tables S2.3-5). However, this fine-scale division corresponds exactly to their geographic sampling ranges (Killick-Kendrick and Peters 1978); Table S2.1). Since the species-delimitation approaches assume random mating within the delimited lineages and that this assumption is not met in the presence of population structure, we suggest that this conclusion be treated with caution (Yang and Rannala 2010). Nevertheless, the extreme divergence between some subspecies, especially those within *P. vinckei* (*P. v. vinckei*, *P. v. brucechwatti* and *P. v. lentum*), clearly warrants careful attention, as these lineages are at least as divergent as *P. berghei* and *P. yoelii* (Table 2.2 and Figures 2.1 and S2.1-2). Treating *P. vinckei* subspecies as species would resolve the inconsistency between level of divergence and taxonomic rank of *P. v. vinckei* first detected in (Perkins et al. 2007). Regardless of taxonomy, these deep divergences need to be taken into careful consideration when considering experimental studies with *P. vinckei*. More broadly, the correct identification of independently evolving lineages (species) is of critical epidemiological relevance, as it can enable interbreeding populations to be identified, which is essential for the management of disease (Lymbery and Thompson 2012).

(Perkins et al. 2007) provided an earlier rodent malaria phylogeny, based on 7 loci and 19 genotypes. Our analyses, based on 11 loci and 58 genotypes, similarly confirms the original morphological and isozyme-based species classification (Killick-Kendrick and Peters 1978). The topology of our species tree broadly matches that of Perkins *et al.* (Perkins et al. 2007), suggesting a robust rodent malaria phylogeny. We used three time-calibrations to estimate the divergence times of rodent *Plasmodium*. The Ricklefs2010

calibration (Ricklefs and Outlaw 2010) results in estimates that are contained within those of the Pacheco2011-B calibration. Thus, given we have little information about the divergence process of rodent malaria parasites or their hosts we consider the full span of the combined 95% HPDs for both Pacheco2011 calibrations as the estimates which best reflect the available knowledge, i.e. the major splits in the rodent phylogeny occurred at roughly 4-18 (basal split; see (Pacheco et al. 2011), 1-6 (*P. berghei – P. yoelii*) and 3-13 Mya (*P. chabaudi – P. vinckei*). These estimates coincide with a recent estimate for the divergence of the *Grammomys* genus (which includes known host species for rodent *Plasmodium*; Lecompte et al. 2008).

Whilst polymorphism was generally low, *P. vinckei* displayed significantly more diversity than other taxa. However, this is because divergence between *P. vinckei*'s subspecies is much greater than for the *P. chabaudi* and *P. yoelii* subspecies. Accordingly, the level of polymorphism within *P. v.* subsp. (the *P. vinckei* subspecies with most available genotypes) is within the range of the other taxa. This suggests that, at least for *P. vinckei*, molecular evolution tools should be applied at the subspecies, rather than the species level, due to the deep divergences of its subspecies. Given this, care should be taken when generalizing observations from a single *P. vinckei* subspecies to the species level.

In contrast to *P. vinckei,* our analysis confirms previous reports that genetic diversity for *P. berghei* is very low, but not zero (Saula et al. 1997; Perkins et al. 2007). Based on the apparently low diversity in *P. berghei*, it has been suggested that the diversity detected in the past could have arisen in the lab (i.e. after isolation). We observed 2 polymorphisms across 6500 observed sites in *P. berghei* (assuming a star-shaped phylogeny for the genotypes). This is much higher than the expected value (0.005 mutations) if these mutations had been acquired after isolation (assuming 60 years since parasite isolation and a mutation rate of 1.2×10^{-8} per site per year; Ricklefs and Outlaw 2010), which suggests that the observed mutations were probably present in the natural populations. Despite *P. berghei*'s key role as a model for the study of functional

genomics (Janse et al. 2006), this near absence of polymorphism limits its relevance for understanding the functional consequences of genetic variation. At the subspecies level, *P. c. chabaudi*, *P. y. yoelii* and *P. v.* subsp. show the highest diversity (in terms of π_S), making these the best rodent models for the identification of genetic markers for quantitative-trait loci analysis. Whilst genotypes of *P. c. chabaudi* and *P. y. yoelii* have been used to identify drug resistance and virulence loci (Hayton et al. 2002; Li et al. 2011), *P. v.* subsp. has not been used in this type of study. However, given that average π_S for this species is twice that of *P. y. yoelii* and almost as high as for *P. c. chabaudi*, this subspecies may provide an alternative model for such studies.

The relative rates of protein evolution (K_A/K_S) did not differ between species/subspecies and suggest that almost all loci analyzed are evolving under some constraint, as would be expected for house-keeping loci. However, some loci do show very low levels of constraint in some lineages (e.g. *rnabind* in *P. chabaudi*), possibly suggesting functional differences between lineages (Figure 2.2, Tables S2.7-8). Accordingly, a comparison of bootstrap intervals failed to detect significant differences between lineages in maximum-likelihood estimates of α, and overall there is little evidence for positive selection. The lack of variation in mean α and K_A/K_S between lineages is perhaps surprising given the apparently large differences seen in effective population size (both in the *BEAST analysis and in terms of π_S; Gossmann et al. 2012). However, this may mostly reflect lack of power, as both the estimates of mean α and K_A/K_S present large confidence intervals (Figure 2.2; Tables S2.7-9). Nevertheless, our mean estimates of α do increase with effective population size (as might be expected if positive selection is more effective at higher effective population sizes; Tables S2.6 and S2.10; Gossmann et al. 2012). Additionally, using individual gene-wise MK analysis, we identified a single potential candidate for the action of strong positive selection: *cyspro* in *P. chabaudi*. As no correction for multiple tests was made, we treat this result with caution. Nevertheless, given that the *P. falciparum* ortholog of *cyspro* (falcipain-1) has been proposed as a possible vaccine candidate (Kumar et al. 2007), if the functional role and selective patterns of *cyspro* are similar in *P. chabaudi* and *P. falciparum*, *P. chabaudi* could be a

useful model to test the efficacy of vaccines targeting this locus and understand potential evolutionary responses by the parasite.

Finally, we show that linkage disequilibrium (LD) is generally low, particularly at the subspecies level for *P. c. chabaudi* and *P. y. yoelii*. Importantly, the low LD means that without knowledge of the parasite's parental genotypes, it may be difficult to infer similarity in trait values from shared genetic markers, unless marker density is very high. For example, with low LD it would be hard to predict a correlation in cross-immunity based on an excess of shared markers, because it is unlikely that those marker alleles will be in LD with the causal locus.

The work here presented improves the utility of rodent *Plasmodium* as models for the study of malaria as it provides a much-needed evolutionary context for understanding biological aspects as the cross-reactivity of immune responses or parasite mating patterns. Moreover, while evolutionary genetics tools have rarely been used to study rodent *Plasmodium*, this approach has great utility for understanding the selective pressures shaping the evolution of malaria parasites and for generating hypotheses about the molecular interactions between parasites, hosts, and vectors.

2.6. APPENDIX

Table S2.1. Summary of the rodent malaria parasite genotypes used.

Table S2.2. Primer sequences (5'-3') used in PCR for the selected loci.

Table S2.3. Posterior probabilities for the speciation models sampled by BPP, using the different algorithms and fine-tune parameters (ε, *a* and *m*), in the 5 and the 11 loci dataset.

Table S2.4. Posterior probabilities for the speciation models sampled by BPP, using different values for the θ prior, in the 5 and the 11 loci dataset.

Table S2.5. Ensemble genealogical sorting index (*gsiT*) for the independently evolving lineages identified by BPP, in the 5 (as in Table S2.3) and the 11 loci dataset.

Table S2.6. Population sizes for the rodent malaria subspecies as inferred by *BEAST.

Table S2.7. Summary of the analysis of polymorphism and divergence in the four species of rodent malaria across the 11 loci (Electronic version only).

Table S2.8. Summary of the analysis of polymorphism and divergence for four of the subspecies of rodent malaria across the 11 loci (Electronic version only).

Table S2.9. Pairwise comparisons of πA, πS and $\pi A/\pi S$ across taxa.

Table S2.10. Best likelihood models and estimates of the proportion of adaptive substitutions across loci ($\bar{\alpha}$).

Figure S2.1. Tree inferred by *BEAST under the Pacheco2011-A calibration for the simple substitution model.

Figure S2.2. Tree inferred by *BEAST under the Pacheco2011-B calibration for the simple substitution model.

Figure S2.3. Linkage Disequilibrium (r^2) for the subspecies *P. c. chabaudi* (left) and *P. y. yoelii* (right).

Figure S2.4. Linkage Disequilibrium (r^2) for the species *P. chabaudi* (left) and *P. yoelii* (right).

Table S2.1. Summary of the rodent malaria parasite genotypes used. Lines in bold represent clones. Location: CAM - Cameroon; CAR - Central African Republic; CON - Congo; DRC - Democratic Republic of the Congo; NIG - Nigeria. Mixed infection: nk - not known; Pcc - *P. c. chabaudi*; Pv - *P. vinckei*; Pvp - *P. v. petteri*; Pyk - *P. y. killicki*; Pysp - *P. y.* subsp.; Pyy - *P. y. yoelii*. ? - information is uncertain. Note that where isolate and arrival dates are the same is because infected *T. rutilans* were shipped to Edinburgh.

species	subsp.	isolate	line (genotype)*	isolate date	arrival date	isolated from	location	mixed infection
P. berghei		ANKA	ANKA	07-03-1965	11-1968	*Anopheles dureni millecampsi*	River Kasapa (DRC)	nk
			ANKAcl5					
		K173	K173	01-1948	02-08-1983	*Garmmomys surdaster*	River Kisanga (DRC)	nk
			K173 (N strain)		04-10-1973			
		KSP11	KSP11	04-11-1961	20-02-1978	*An. d. millecampsi*	Katanga (DRC)	nk
		LUKA	LUKA	15-03-1966	02-10-1971		River Lukoma, Kamena (DRC)	nk
		NK65	NK65	04-01-1964	15-10-1968		River Kisanga (DRC)	nk
		SP11	SP11	02-1961	11-1971		River Kasapa (DRC)	nk

Table S2.1 (cont.)

species	subsp.	isolate	line (genotype)*	isolate date	arrival date	isolated from	location	mixed infection
P. chabaudi	*adami*	556KA	**19DK1**	1970	04-12-71	*Thamnomys rutilans*	N'ganga Lingolo (CON)	nk
			19DK23		04-12-71			
	chabaudi	AD	**AD**	19-03-1969	19-03-1969	*T. rutilans*	Maboké Field Station (CAR)	yes (Pyy)
		AJ	**AJ**	19-03-1969	19-03-1969			yes (Pvp)
		AL	**AL**	18-04-1969	18-04-1969			no
		AQ	**AQ**	18-04-1969	18-04-1969			no
		AS	**ASsens**	18-04-1969	18-04-1969			no
		AT	**AT**	26-03-1969	26-03-1969			no
		BK	**BK**	18-04-1969	18-04-1969			no
		BS	**4BS20**	25-09-1970	25-09-1970			yes (Pvp)
		CB	**CB**	25-09-1970	25-09-1970			no
		CE	**13CE11**	25-09-1970	25-09-1970			yes (Pvp)
		CQ	**CQ**	25-09-1970	25-09-1970			yes (Pvp)
		CW	**CW**	25-09-1970	25-09-1970			yes (Pyy)
		ER (=56L)	**8ER12**	04-1965	12-1983			nk
		v-52	**v-52#**	?	?			*?*

Table S2.1 (cont.)

species	subsp.	isolate	line (genotype)*	isolate date	arrival date	isolated from	location	mixed infection
P. vinckei	*brucechwatti*	1/69β	1/69β	1969	23-07-1970	*T. rutilans*	Ilobi Village (NIG)	nk
		N48 (DB)	N48 (DB)	09-1967	25-10-1971			nk
	lentum	170L (DE)	170L (DE)	07-1966	25-10-1971	*T. rutilans*	N'ganga Lingolo (CON)	nk
		194ZZ (DJ)	194ZZ (DJ)	09-1966	24-12-1971			yes (Pyk)
		483L (DD)	483L (DD)	07-1966	25-10-1971			nk
	petteri	BS	**4BS19**	25-09-1970	25-09-1970	*T. rutilans*	Maboké Field Station (CAR)	yes (Pcc)
			4BS2					
		BZ	BZ	25-09-1970	25-09-1970			nk
	vinckei	v-67?	VIBA Cy0	24-01-1967?	02/10/1971?	*An. d. millecampsi*	River Lukoma, Kamena (DRC)	nk
			VIBA CyPl					
	subsp.	Biboto 18(EE)	Biboto 18(EE)	08-1973	24-10-1977	*T. rutilans*	Eseka? (CAM)	no
		Esekam II	**31EH**	03-1974	24-10-1977			yes (Pysp)
		Esekam III	EK	03-1974	10-05-1978			no
		Esekam IV	**10EL2**	03-1974	24-10-1977			yes (Pysp)
			8EL15					

Table S2.1 (cont.)

species	subsp.	isolate	line (genotype)*	isolate date	arrival date	isolated from	location	mixed infection
P. yoelii	*killicki*	193L	314ZZ?	09-1966	10-1971	*T. rutilans*	N'ganga Lingolo (CON)	nk
		194ZZ		28-10-1968	25-11-1968			nk
	nigeriensis	N67	**212D**	08-1967	1968	*T. rutilans*	Ilobi Village (NIG)	nk
		v-67	v-67#	?	?	?	?	?
	yoelii	17X	**A**	04-1965	12-1967	*T. rutilans*	Maboké Field Station (CAR)	nk
			Mill Hill					
		32X	32X	1965	1968			nk
		33X	**C**	04-1965	16-05-1968			nk
		55X	55X	1965	1968			yes (Pcc)
		86X	86X	04-1965	1968			yes (Pcc)
		146X	146X	04-1965	1968			no
		AR	**56AR18**	26-03-1969	26-03-1969			yes (Pcc)
		CP	CP	25-09-1970	25-09-1970			yes (Pcc, Pvp)
		CU	**23CU12**	25-09-1970	25-09-1970			no

Table S2.1 (cont.)

species	subsp.	isolate	line (genotype)*	isolate date	arrival date	isolated from	location	mixed infection
P. yoelii	*subsp.*	Esekam II	EH	03-1974	24-10-1977	*T. rutilans*	Eseka? (CAM)	yes (Pv)
		Esekam IV (EL)	**10EL1**	03-1974	24-10-1977			yes (Pv)
		Esekam IV (EM)	EM	03-1974	24-10-1977			yes (Pv)

Notes:

*We define parasite isolate, line and clone as in (Killick-Kendrick and Peters 1978; in the main text). Briefly, an 'isolate' is a sample of parasites collected from a unique animal on a single occasion, potentially including multiple genetically distinct lineages. 'Lines' are experimentally manipulated isolates that may retain the original genetic diversity. 'Clones' are derived from a single haploid parasite and are genetically uniform. Because we either used different isolates or clones, here we refer to the different parasites as 'genotypes'.

#As stated in the main text *P. c. chabaudi* v-52 and *P. y.nigeriensis* v-67 were previously mislabelled as *P. v. vinckei*. Therefore, we removed any information associated with this, as it is irrelevant.

Table S2.2. Primer sequences (5'-3') used in PCR for the selected loci. PCR cycling conditions: 95ºC for 3 min.; 10 cycles of: 94ºC for 30s, 57ºC for 30s (-1ºC per cycle), 72ºC for 1.5 min.; 35 cycles of: 94ºC for 30s, 47ºC for 30s, 72ºC for 1.5 min.; 72ºC for 3 min.

gene	forward primer	reverse primer
26s	ATCATCAGGGGAACATTTACCATCGA	ATGCCATTCCTTCAACTGTGGCAAT
atpase	ATTGATACTCCTGGACAAATAGAA	TGACATCTTTGTCTTCTCCATATTG
cons	TGATCACAGTTTAAGTGTATATGACAG	TTTCTCTGCCTTTATCATGGTAC
cyspro	CAATGAAATGGTAGGTAAAAATGGT	CCATGAATTTCTAATGATCCAGTAAT
dhfr	CAAGATGATAGAACAGGTGTTGGTG	CCCAATACATGTATAAATTCAGCTG
exonuc	GTAGTGCGTCCTATGGATATTTA	GAATTAGCTCTTTTGATATCGGTCT
gdpgmp	TGAAGGGATAAAACATTTATGGCCT	GTGTATTGCTTGTCCTATGGAAACG
glurna	AAGTTGTTACTCGATTTCCACC	GGCATGCAAAATCATAAGTTGGAT
hsp70	TTCCTATGATTATGTTGTGGATCA	CGTGTAACTGTTTCTTGTAAGC
metrans	TATTACCAGCAAGTTTTAGAGC	GGAATTGTACCAGTAAATCCTT
rnabind	GGAAAGTATGTCTAGGTATTCCAAC	GGTCTGCTATCTCCTAATTGTGA

Table S2.3. Posterior probabilities for the speciation models sampled by BPP, using the different algorithms and fine-tune parameters (ε, *a* and *m*), in the 5 and the 11 loci dataset. Priors were kept constant: θ~G(1.5,300) and τ_0~G(1.5,30). 5 loci dataset: *26s*, *atpase*, *dhfr*, *exonuc*, *rnabind*. Each speciation model is represented using 0-1 flags for the interior nodes, with 0 indicating a collapsed node and 1 a resolved node. Nodes are ordered as in Figure 2.1, with the first flag corresponding to node *a*, the second to node *b*, and so on up to node *l*. Each cell has the posterior probability for two replicate runs of BPP, which started with different speciation models. NS: model not sampled.

dataset	speciation model	algorithm 0		algorithm 1			
		ε = 5	**ε = 20**	***a* = 0.5; *m* = 1**	***a* = 0.5; *m* = 2**	***a* = 2; *m* = 1**	***a* = 2; *m* = 2**
5 loci	111111111111	0.958; 0.955	0.954; 0.965	0.956; 0.965	0.951; 0.956	0.960; 0.962	0.962; 0.960
	111101111111	0.041; 0.044	0.045; 0.035	0.043; 0.035	0.048; 0.042	0.039; 0.037	0.038; 0.039
	111111101111	6.1E-4; 1.4E-3	7.3E-4; 2.3E-4	1.1E-3; 6.2E-4	1.5E-3; 1.6E-3	1.1E-3; 1.0E-3	5.4E-4; 8.4E-4
	111111111110	3E-5; 2.8E-5	3E-6; NS	2.7E-5; 2.1E-5	1.7E-5; 2E-6	1.5E-5; 3.7E-5	1.6E-5; 2.2E-5
	111101101111	2.8E-5; NS	NS	NS	5.4E-5; 5.7E-5	NS	NS
	111101111110	NS; 8E-6	NS	1E-6; 1E-6	NS	NS	NS
11 loci	111111111111	0.997; 0.996	1; 1	0.996; 0.991	0.998; 1	0.995; 0.999	0.994; 0.986
	111101111111	2.7E-3; 3.7E-3	NS	4.1E-3; 8.7E-3	2.1E-3; NS	5.5E-3; 1.2E-3	6.2E-3; 0.014

Table S2.4. Posterior probabilities for the speciation models sampled by BPP, using different values for the θ prior, in the 5 and the 11 loci dataset. We varied only the θ prior because, contrary to the τ_0 prior, this has been shown to have a strong impact upon the speciation probabilities (Leache and Fujita 2010; Yang and Rannala 2010; Zhang et al. 2011). Additionally, to test for the robustness of our results, we used performed this analysis with the entire dataset (11 loci) or with a reduced dataset (5 loci: *26s*, *atpase*, *dhfr*, *exonuc*, *rnabind*). We used algorithm 0 with fine-tune ε = 5 for this analysis. Speciation model and cell content are as described for Table S2.3.

dataset	speciation model	G(1.5, 1500)	G(1.5, 150)
5 loci	111111111111	0.871; 0.855	0.931; 0.942
	111101111111	0.105; 0.112	0.068; 0.057
	111111101111	0.020; 0.029	1.1E-3; 9.06E-3
	111111111110	8E-6; 8E-6	8.5E-5; 9.6E-5
	111101101111	3.5E-3; 3.0E-3	6.4E-5; 1.2E-4
	111101101110	3E-6; NS	NS
	111101111110	NS	NS;9E-6
11 loci	111111111111	0.939; 0.939	0.988; 0.975
	111101111111	0.010; 0.005	0.012; 0.025
	111111101111	0.050; 0.061	NS

Table S2.5. Ensemble genealogical sorting index (*gsiT*) for the independently evolving lineages identified by BPP, in the 5 (as in Table S2.3) and the 11 loci dataset. Each cell has the *gsiT* and the associated *p-values* (in brackets).

species	dataset	
	5 genes	**11 genes**
P. berghei	1.0000 (<0.001)	0.8333 (<0.001)
P. c. adami	0.4000 (<0.001)	0.4433 (<0.001)
P. c. chabaudi	0.7898 (<0.001)	0.8464 (<0.001)
P. c. subsp.	0.5992 (0.0136)	0.4163 (<0.001)
P. v. brucechwatti	0.8000 (<0.001)	0.9167 (<0.001)
P. v. lentum	1.0000 (<0.001)	0.9167 (<0.001)
P. v. petteri	0.9308 (<0.001)	0.7407 (<0.001)
P. v. subsp.	0.7553 (<0.001)	0.7855 (<0.001)
P. v. vinckei	1.0000 (<0.001)	0.9167 (<0.001)
P. y. killicki	0.8000 (0.0224)	0.5986 (0.0954)
P. y. nigeriensis	0.8000 (<0.001)	0.6667 (<0.001)
P. y. subsp.	0.3217 (0.0141)	0.3308 (0.0854)
P. y. yoelii	0.7214 (<0.001)	0.7613 (<0.001)

Table S2.6. Population sizes for the rodent malaria subspecies as inferred by *BEAST. Point estimates (and 95% HPDs) are shown for each calibration for start and end population size. Pb - *P. berghei*; Pca - *P. c. adami*; Pcc - *P. c. chabaudi*; Pcsp - *P. c.* subsp.; Pvb - *P. v. brucechwatti*; Pvl - *P. v. lentum*; Pvp - *P. v. petteri*; Pvsp - *P. v.* subsp.; Pvv - *P. v. vinckei*; Pyk - *P. y. killicki*; Pyn - *P. y. nigeriensis*; Pysp - *P. y.* subsp.; Pyy - *P. y. yoelii*.

population size	taxa	Pacheco2011-A	Pacheco2011-B
start	Pb	3.19E+05 (9.67E+04; 6.20E+05)	5.71E+05 (1.95E+05; 1.06E+06)
	Pca	2.07E+06 (2.84E+05; 4.41E+06)	3.73E+06 (6.96E+05; 7.76E+06)
	Pcc	9.25E+06 (3.75E+06; 1.58E+07)	1.66E+07 (8.46E+06; 2.71E+07)
	Pcsp	1.38E+06 (8.76E+04; 3.29E+06)	2.47E+06 (2.56E+05; 5.92E+06)
	Pvb	8.60E+05 (1.28E+05; 1.92E+06)	1.53E+06 (2.67E+05; 3.27E+06)
	Pvl	8.86E+05 (2.00E+05; 1.78E+06)	1.60E+06 (4.38E+05; 3.15E+06)
	Pvp	1.24E+06 (2.34E+05; 2.66E+06)	2.24E+06 (4.90E+05; 4.74E+06)
	Pvsp	1.89E+06 (3.98E+05; 3.81E+06)	3.40E+06 (8.45E+05; 6.65E+06)
	Pvv	4.16E+05 (4.32E+04; 9.92E+05)	7.43E+05 (5.38E+04; 1.70E+06)
	Pyk	1.19E+06 (1.36E+05; 2.74E+06)	2.15E+06 (2.69E+05; 4.88E+06)
	Pyn	8.79E+05 (6.06E+04; 2.15E+06)	1.58E+06 (1.67E+05; 3.80E+06)
	Pysp	2.01E+06 (1.90E+05; 4.70E+06)	3.60E+06 (3.56E+05; 8.29E+06)
	Pyy	2.04E+06 (5.52E+05; 4.07E+06)	3.70E+06 (1.05E+06; 6.89E+06)

Table S2.6 (cont.)

population size	taxa	Pacheco2011-A	Pacheco2011-B
end	Pb	5.61E+05 (1.76E+05; 1.05E+06)	2.58E+06 (1.93E+05; 6.41E+06)
	Pca	1.81E+06 (5.35E+05; 3.48E+06)	2.93E+06 (1.86E+05; 5.93E+06)
	Pcc	4.42E+06 (9.64E+05; 8.46E+06)	6.54E+06 (8.40E+05; 1.48E+07)
	Pcsp	8.74E+05 (1.52E+04; 2.09E+06)	1.47E+06 (3.12E+03; 3.83E+06)
	Pvb	9.44E+05 (2.14E+05; 1.96E+06)	2.24E+06 (3.42E+05; 4.89E+06)
	Pvl	9.33E+05 (2.85E+05; 1.79E+06)	1.93E+06 (3.16E+05; 4.20E+06)
	Pvp	9.29E+05 (5.75E+04; 1.96E+06)	1.50E+06 (1.56E+04; 3.55E+06)
	Pvsp	2.70E+06 (1.05E+06; 4.74E+06)	5.21E+06 (1.95E+06; 8.87E+06)
	Pvv	5.10E+05 (7.72E+04; 1.09E+06)	1.52E+06 (1.71E+05; 3.51E+06)
	Pyk	1.03E+06 (2.12E+05; 2.21E+06)	1.67E+06 (3.54E+04; 3.89E+06)
	Pyn	8.54E+05 (1.06E+05; 1.94E+06)	1.51E+06 (4.84E+04; 3.81E+06)
	Pysp	6.05E+05 (2.42E+03; 1.62E+06)	1.08E+06 (1.62E+04; 2.82E+06)
	Pyy	9.58E+05 (1.30E+05; 2.07E+06)	1.70E+06 (2.34E+05; 3.58E+06)

Table S2.9. Pairwise comparisons of πA, πS and $\pi A/\pi S$ across taxa. *p-values* for pairwise Wilcoxon tests with Bonferroni corrections are shown. $p < 0.05$ are highlighted in bold. b - *P. berghei*, c - *P. chabaudi*, cc - *P. c. chabaudi*, v - *P. vinckei*, vp - *P. v. petteri*, y - *P. yoelii*, yy - *P. y. yoelii*.

statistic	taxa	b	c	cc	v	vp	vsp	y
πA	c	0.21	-	-	-	-	-	-
πA	cc	0.56	1.00	-	-	-	-	-
πA	v	**0.00**	0.24	**0.03**	-	-	-	-
πA	vp	1.00	0.08	0.18	**0.01**	-	-	-
πA	vsp	1.00	1.00	1.00	0.23	0.76	-	-
πA	y	0.58	1.00	1.00	1.00	0.38	1.00	-
πA	yy	1.00	1.00	1.00	0.07	0.76	1.00	1.00
πS	c	**0.00**	-	-	-	-	-	-
πS	cc	**0.00**	1.00	-	-	-	-	-
πS	v	**0.00**	0.19	0.07	-	-	-	-
πS	vp	1.00	0.23	0.23	**0.03**	-	-	-
πS	vsp	**0.02**	1.00	1.00	0.16	0.84	-	-
πS	y	**0.00**	1.00	1.00	**0.03**	0.20	1.00	-
πS	yy	**0.01**	0.23	0.29	**0.00**	1.00	1.00	0.79
$\pi A/\pi S$	c	**0.04**	-	-	-	-	-	-
$\pi A/\pi S$	cc	0.10	1.00	-	-	-	-	-
$\pi A/\pi S$	v	**0.01**	1.00	1.00	-	-	-	-
$\pi A/\pi S$	vp	-	0.07	0.17	**0.01**	-	-	-
$\pi A/\pi S$	vsp	0.76	1.00	1.00	1.00	1.00	-	-
$\pi A/\pi S$	y	0.23	1.00	1.00	1.00	0.36	1.00	-
$\pi A/\pi S$	yy	0.76	1.00	1.00	1.00	1.00	1.00	1.00

Table S2.10. Best likelihood models and estimates of the proportion of adaptive substitutions across loci ($\bar{\alpha}$). Divergence was measured to the inferred ancestral sequence of the *P. chabaudi-P. vinckei* clade (*P. c. chabaudi* and *P. v.* subsp.) or the *P. berghei-P. yoelii* clade (*P. y. yoelii*). The best (highest Akaike weight) and secondary models are shown, with Akaike weights in brackets. Estimates of $\bar{\alpha}$ (and 95% CI) across loci were obtained from 1000 bootstraps on the results of model ii. Likelihood models: i) α is constrained to zero at all loci, i.e. no adaptive evolution; ii) α is a free-parameter common to all loci; and iii) α can take a different value at each locus.

taxa	**dataset**	**best model**	**secondary model**	$\bar{\alpha}$
P. c. chabaudi	all loci	iii (1.00)	-	0.640 (0.212; 0.874)
	w/out *cyspro*	ii (0.65)	i (0.33)	0.456 (0.061; 0.769)
P. vinckei subsp.	all loci	iii (0.97)	i (0.03)	0.165 (-0.120; 0.516)
	w/out *cyspro*	i (0.73)	ii (0.27)	0.279 (-0.067; 0.698)
P. y. yoelii	all loci	i (0.74)	ii (0.26)	-0.261 (-1.189; 0.516)
	w/out *cyspro*	i (0.70)	ii (0.30)	-0.524 (-1.939; 0.470)

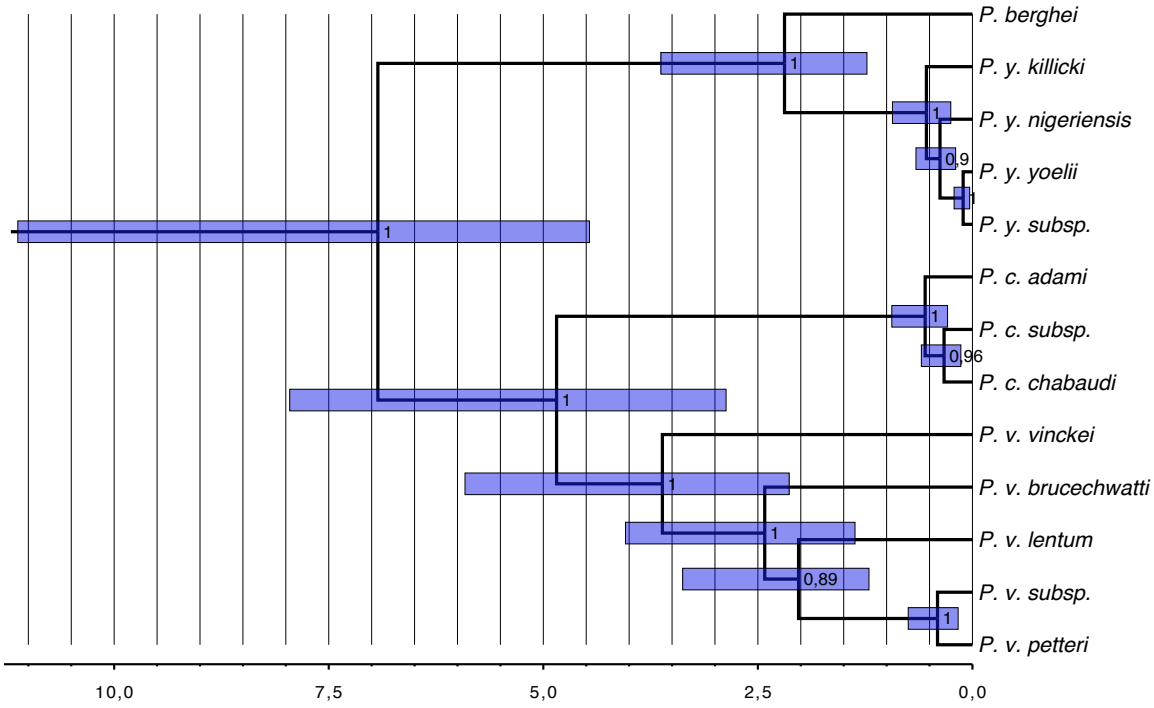

Figure S2.1. Tree inferred by *BEAST under the Pacheco2011-A calibration for the simple substitution model. Tree was calibrated with a gamma-distributed prior for the root of *coI* and *cytb*, with shape = 3.216578, scale = 1.258366 and offset = 4.272368. Node labels are posterior probabilities and node bars represent 95% Highest Posterior Densities on the height of each node. Axis is in million years ago (Mya).

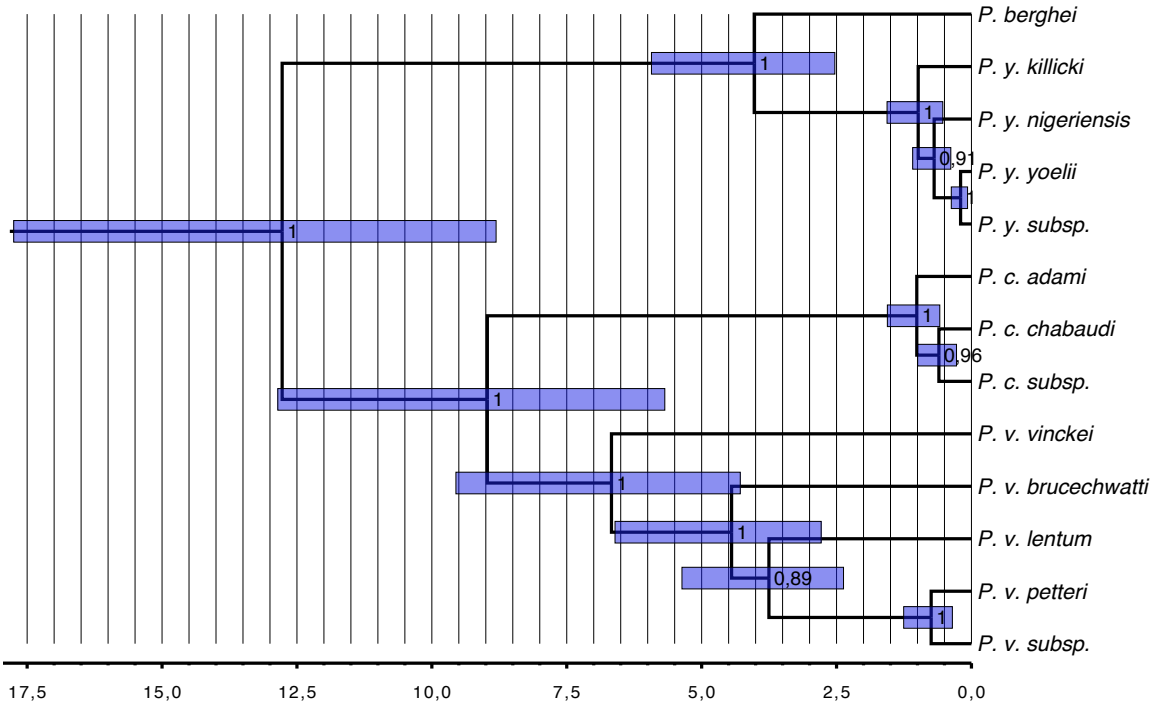

Figure S2.2. Tree inferred by *BEAST under the Pacheco2011-B calibration for the simple substitution model. Tree was calibrated with a gamma-distributed prior for the root of *coI* and *cytb*, with shape = 7.607868, scale = 0.9322701 and offset = 7.1074117. Node labels are posterior probabilities and node bars represent 95% Highest Posterior Densities on the height of each node. Axis is in million years ago (Mya).

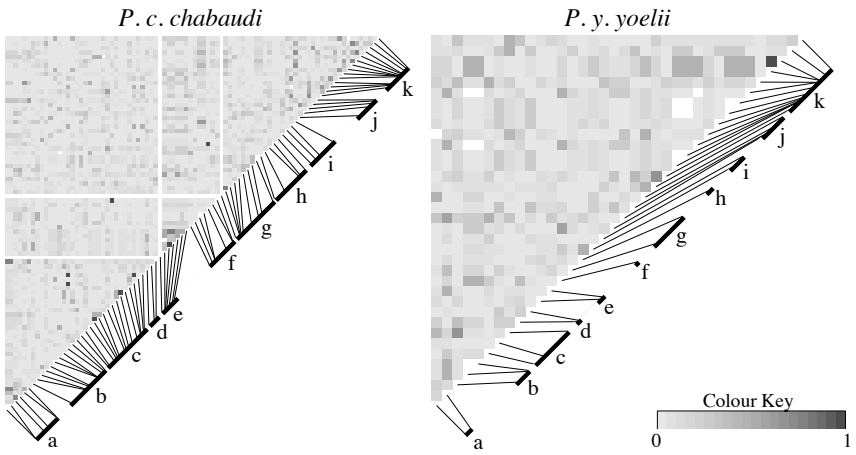

Figure S2.3. Linkage Disequilibrium (r^2) for the subspecies *P. c. chabaudi* (left) and *P. y. yoelii* (right). Genes within a chromosome are ordered by increasing distance. Genes a-e correspond to chromosome 3; f – chromosome 7; and g-k correspond to chromosome 13. a - *exonuc*; b – *26s*; c – *metrans*; d – *gdpgmp*; e – *cons2*; f – *dhfr;* g – *cyspro*; h – *rnabind*; i – *hsp70*; j – *glurna*; k – *atpase*.

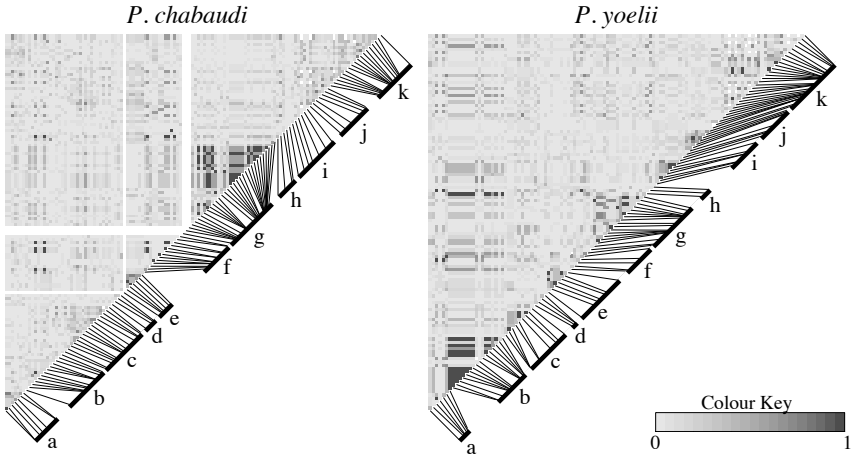

Figure S2.4. Linkage Disequilibrium (r^2) for the species *P. chabaudi* (left) and *P. yoelii* (right). Genes within a chromosome are ordered by increasing distance. Genes a-e correspond to chromosome 3; f – chromosome 7; and g-k correspond to chromosome 13. a - *exonuc*; b – *26s*; c – *metrans*; d – *gdpgmp*; e – *cons2*; f – *dhfr;* g – *cyspro*; h – *rnabind*; i – *hsp70*; j – *glurna*; k – *atpase*.

3. Sex and species recognition in *Plasmodium*

3.1. SUMMARY

To transmit to new hosts, malaria parasites must undergo a round of sexual reproduction in a mosquito vector. Remarkably little is known about the ecology of mating and the molecular interactions that occur during fertilisation in malaria parasites. Therefore, basic questions, such as: 'Do different *Plasmodium* species from mixed-species infections hybridise?' remain unanswered. We used genetically modified rodent malaria parasites to test whether hybridisation can occur between *P. berghei* and *P. yoelii* and whether pre-zygotic hybridisation barriers exist. We demonstrate that malaria parasites can hybridise, but that this only occurs at high levels (i.e. little discrimination between con- and heterospecifics) when either of the proteins P230 and P48/45 are absent from the surface of female gametes, suggesting these proteins mediate mate recognition. We also show asymmetric reproductive interference, in which *P. berghei* fertility is reduced by the presence of *P. yoelii*, but not vice-versa. In metazoan organisms, genes involved in mate recognition often evolve under positive selection, so we tested if this also applies to unicellular malaria parasites. We collected sequence data for P230, P48/45 and P47 (P48/45 paralog), from all rodent malaria parasite species and subspecies, and we found strong positive selection for some of these loci: particularly for P47 and a region of P230. Given that P230 and P48/45 are two of the main targets for the development of transmission-blocking vaccines, our results may have applied implications.

3.2. INTRODUCTION

The maintenance of species identity and the process of speciation require reproductive barriers to genetic exchange between species (Coyne and Orr 2004; Palumbi 2009). These barriers can be divided into pre- and post-zygotic (Coyne and Orr 2004). Pre-zygotic barriers usually involve ecological (e.g. habitat choice) or recognition processes (e.g. preference for con-specific mates, species-specific gamete interactions) and post-zygotic barriers include hybrid sterility or inviability (Coyne and Orr 2004). Whilst different barriers tend to be studied in isolation, they act throughout the life-cycle of an organism, with each barrier further reducing the gene flow that escaped previous barriers (Coyne and Orr 1998; Matsubayashi and Katakura 2009).

In malaria (*Plasmodium*) parasites, the mechanisms leading to the origin and maintenance reproductive isolation have largely been ignored. To be transmitted between vertebrate hosts, malaria parasites must undergo a round of sexual reproduction in the blood meal of an insect vector (Alano and Carter 1990). To achieve this, developmentally arrested male and female sexual stages (termed gametocytes) are produced throughout infections. As soon as they are taken up in a mosquito blood meal, gametocytes have a short time window (30-60 minutes) to differentiate into gametes (gametogenesis) and achieve fertilisation (Sinden and Croll 1975; Alano and Carter 1990; Talman et al. 2004). During gametogenesis, each female gametocyte differentiates into a single gamete, but male gametogenesis (termed exflagellation) culminates in the production of up to eight 'sperm-like' gametes (Sinden and Croll 1975; Sinden 1983; Janse et al. 1986; Sinden 1998; Reece et al. 2008). Post-fertilisation, zygotes take 18-20 hours to develop into stages that are infective to the vector (ookinetes) (Alano and Carter 1990; Vaughan 2007). The cues that stimulate gametogenesis are conserved across *Plasmodium* species and involve a drop in temperature (>5 ºC), xanthurenic acid, and unidentified host factors (Nijhout and Carter 1978; Sinden et al. 1996; Billker et al. 1997; Billker et al. 1998; Garcia et al. 1998; Billker et al. 2000; Arai et al. 2001).

All gametocytes in a blood meal mate in an extremely constrained space and time-window, so heterospecific gamete interactions can only occur if mixed-species infections exist in vertebrate hosts and concurrently produce gametocytes that are co-transmitted to mosquitoes during blood feeding. Field and lab data demonstrate these scenarios do occur. First, multiple species of *Plasmodium* naturally infect the same hosts, including humans (*Plasmodium falciparum*, *P. knowlesi*, *P. malariae*, *P. ovale* and *P. vivax*) and thicket rats (*P. berghei*, *P. chabaudi*, *P. vinckei* and *P. yoelii*) (Killick-Kendrick and Peters 1978; Mayxay et al. 2004; Singh et al. 2004). Second, mixed-species infections are common in humans (e.g. 55-65% by PCR in Laos and Thailand; Mayxay et al. 2004) and were present in ~28% of the original rodent *Plasmodium* isolates (see Table S2.1). Third, a number of *Anopheles* species are capable of transmitting more than one *Plasmodium* species (McKenzie and Bossert 1997), with mixed-species infections found in wild captured mosquitoes (sometimes at higher prevalence than in humans of the same area; McKenzie and Bossert 1997). Mosquitoes can also simultaneously acquire and transmit multiple-species in experimental conditions (McKenzie and Bossert 1997; Imwong et al. 2011). Furthermore, mixed-species mosquito infections may have an important impact on transmission, as reproductive interference has been shown between two species of avian *Plasmodium* (i.e. fertilisation and transmission success is reduced in mixed- relative to single-species infections; Paul et al. 2002; Groning and Hochkirch 2008).

Therefore conditions for heterospecific gamete interactions exist for malaria parasites, so do pre-zygotic barriers maintain reproductive isolation? During fertilisation, the interaction between male and female gametes generally involves attachment and recognition (potentially at the same time), followed by fusion, and pre-zygotic reproductive barriers can operate at any of these stages (Vacquier 1998; Vieira and Miller 2006). Nevertheless, in organisms with external fertilisation where gametes from multiple species often interact (as in sea urchins or abalone), gamete recognition proteins

have been shown to play a major role in reproductive isolation (Swanson and Vacquier 2002; Palumbi 2009).

It is unknown if malaria parasites have gamete proteins that mediate reproductive isolation, but the following proteins are known to be important for conspecific male-female interactions during the early stages of fertilisation: HAP2/GCS1, P230, P47, and P48/45. HAP2/GCS1 is expressed at the surface of male, but not female gametes, and is required for fusion, but not attachment (Hirai et al. 2008; Liu et al. 2008). P230, P47 and P48/45 belong to the 6-cys multi-domain protein family, whose members are expressed at the surface of gametocytes/gametes or invasive stages (sporozoites and merozoites), suggesting possible roles in cell-cell interactions (Gerloff et al. 2005; van Dijk et al. 2010). P230 and P48/45 are known targets of transmission-blocking immunity (Healer et al. 1999; Bousema et al. 2010) and are expressed at the surface of both male and female gametes, but deletion of either in *P. berghei* only renders males infertile, due to failure to recognize and/or attach to female gametes (Bustamante et al. 2000; van Dijk et al. 2001; Khan et al. 2005; Eksi et al. 2006; van Schaijk et al. 2006; van Dijk et al. 2010). P47 is only expressed at the surface of female gametes and its deletion in *P. berghei* prevents viable male gametes from attaching to females (van Schaijk et al. 2006; van Dijk et al. 2010). Interestingly it has also been suggested that P230, P47, and P48/45 evolve under positive Darwinian selection (van Dijk et al. 2010), which is a common feature of genes involved in gamete recognition (Swanson and Vacquier 2002), making these potential candidates for mediating reproductive isolation.

Here, we test whether pre-zygotic reproductive barriers exist that prevent hybridisation between different species of rodent malaria parasites. Contrary to conventional wisdom, our results show that malaria parasites can hybridise and that this occurs at very high levels when P230 or P48/45 are absent from the surface of female gametes, indicating that P230 and P48/45 are the ligands in females involved in gamete recognition. However, the absence of these proteins does not lead to full random mating between

con- and heterospecifics, suggesting that other, yet unidentified, factors also contribute to recognition. We also demonstrate that asymmetrical reproductive interference occurs. Genes involved in gamete recognition are amongst the most rapidly evolving so we collected and analyzed sequence data for P230, P48/45 and P47 from 58 genotypes of the 4 species of rodent *Plasmodium*. We find strong positive selection acting on P47 and a region of P230, identify specific codons in each gene that experience strong selection and explore the ecological factors that may lead to this.

3.3. MATERIAL AND METHODS

3.3.1. Hosts and parasites

We carried out two sets of *in vitro* experiments using two sister species of rodent malaria parasites, *P. berghei* and *P. yoelii*. Infections were initiated by intra-peritoneal inoculation of parasitized red blood cells (RBCs) in 100 µl carrier solution as in (Bell et al. 2006). We maintained male MF1 mice, aged 8-12 weeks (Harlan-Olac, UK; or in house supplier, University of Edinburgh), on *ad libitum* food (RM3(P), DBM Scotland Ltd, UK) and water (0.05% PABA to enhance parasite growth), with a 12 hour light cycle, at 21 ºC. Two to four days before infection, we treated mice with phenylhydrazine (PHZ) because the resulting release of young RBCs leads to elevated gametocyte production (Gautret et al. 1997).

3.3.2. Overview of experimental design

We harvested gametocytes from mice carrying high numbers of *P. berghei* or *P. yoelii* and mixed them in culture conditions mimicking the vector, thereby inducing gametogenesis and fertilisation (Janse et al 1985), resulting in the production of ookinetes. To distinguish ookinetes resulting from con- or heterospecific fertilisations we blocked the development of male gametocytes into gametes alternatively in each species, so that in each experimental culture we would only have viable male gametes

from a single species. Additionally, we used genetically modified parasite strains whose female gametes and ookinetes (equivalent to zygotes) express fluorescent proteins of different colours (see Figure 3.1). This allowed us to clearly identify whether *P. berghei* or *P. yoeliii* females had been fertilised.

Using this approach, we performed two sets of experiments. First, we tested for reciprocal reproductive barriers between 'intact' (non knock-out) lines of *P. berghei* and *P. yoelii*. Second, we used lines of *P. berghei* in which either P230 *(PbΔp230)* or P48/45 *(PbΔp48/45)* were deleted. A list of lines and their phenotypes is given in Table 3.1 and the number of infections used for each culture type and experiment is given in Table 3.2. We also used the data generated by these experiments to investigate whether the density of con- and heterospecific females affects the level of hybridisation and if there is reproductive interference between species (i.e. if conspecific fertilisation is less successful in mixed- than in single-species cultures; Groning and Hochkirch 2008).

Table 3.1 Parasite lines and phenotypes used in experiments 1 and 2.

species	genetic background	line	aliases	experiment	phenotype
P. berghei	ANKA	820	*PbRFP*	1	RFP females and GFP males; RFP ookinetes
		wild-type	-	2	wild-type
		Δp230	*PbΔp230*	2	P230 knock-out; males unviable; constitutive GFP
		Δp47	-	1	P47 knock-out; females unviable; constitutive GFP
		Δp48/45	*PbΔp48/45*	2	P48/45 knock-out; males unviable; constitutive GFP
P. yoelii yoelii	17X	*GFP*	*PyGFP*	1	constitutive GFP
		wild-type	-	1	wild-type
	33X	*wild-type*	*PyWT*	2	wild-type

3.3.3. Culture conditions

We performed all the experiments *in-vitro* using previously reported conditions (Janse et al. 1985; Reece et al. 2008; Ramiro et al. 2011). Briefly, we harvested parasites (day 3 or 4 post-infection; PI), and incubated them in RPMI (Roswell Park Memorial Institute) media with 10% calf serum at pH 8, at 21 ºC. This mimics the vector environment, immediately triggering gametogenesis, fertilisation and ookinete development. Each experimental culture contained parasites from 2 different species and so we paired mice infected with different species such that each pair of infections contributed parasites to all treatments (mixed-species cultures and controls; see below) and each *P. berghei* infection was paired only with a single *P. yoelii* infection (to avoid pseudo-replication) (Reece et al. 2008; Ramiro et al. 2011). 18-20 hours after incubation, we assayed the numbers of ookinetes (which have a distinctive banana shape), through fluorescence- or light-microscopy, using a haemocytometer. Additionally, on the day of culturing we counted the number of females and exflagellating males present in each infection, as described in (Reece et al. 2008; Ramiro et al. 2011). We assayed ookinetes instead of zygotes, as it is not possible to distinguish zygotes from unfertilised female gametes directly in culture. However, given that zygote development to ookinete and early oocyst is assured by female derived proteins, most of which exist as translationally repressed RNAs (Mair et al. 2006; Mair et al. 2010), it is fair to assume that zygotes resulting from con- or heterospecific matings are just as likely to develop into ookinetes.

3.3.4. Experiment 1: Reproductive Barriers

For experiment 1, we inoculated mice with 10^7 parasitized RBCs of the *P. berghei* lines *PbRFP* or *P. berghei Δp47* (PHZ: 125 mg/kg, day -2PI) or 10^8 parasitized RBC of the *P. y. yoelii* lines *PyGFP* or *P. yoelii* 17X wild-type (PHZ: 60 mg/kg, day -3PI). The different parasite and PHZ dose combinations were used to ensure high gametocyte densities within a few days of infection, so that the presence of transmission blocking immune factors was minimized. We mixed these lines to create test cultures and their controls as described below.

3.3.4.1. Mixed-species cultures

We set up reciprocal crosses between *PbRFP* and *PyGFP* whose female gametes/ookinetes express red fluorescent protein (RFP) and green fluorescent protein (GFP), respectively. To render male gametes of one species unviable, we incubated 15µl of *PbRFP* or *PyGFP* infected blood in 1ml culture media for 12 min. with $5x10^{-4}$ M aphidicolin (Sigma-Aldrich, UK), a specific inhibitor of DNA polymerase-α (Ikegami et al. 1978; Janse et al. 1986). Male gametocytes, but not females, have to synthesize DNA to become gametes so aphidicolin stops male gametogenesis while leaving females unaffected (Janse et al. 1986; Janse et al. 1987; van Dijk et al. 2001). After incubation we washed the aphidicolin off by spinning cultures down (12000 rpm, 5 s), removing the supernatant (parasites remained in the pellet) and adding new culture media (without aphidicolin). During aphidicolin treatment, we collected parasites from all infected mice and added 60µl of *PbRFP* or *PyGFP* infected blood to 4 ml cultures. Finally, we added the 1ml cultures with aphidicolin inactivated males to the 4 ml cultures containing both viable males and females from the other parasite species and assayed the resulting densities and phenotypes of ookinetes. For example, a culture could contain 15µl of aphidicolin treated *PbRFP* (donates viable RFP females) and 60µl of *PyGFP* (donates viable male and GFP females), such that a GFP ookinete results from a *PyGFP* self-fertilisation and an RFP ookinete results from mating between *PyGFP* males and *PbRFP* females (see Figure 3.1 for a schematic description). We used different volumes of blood to provide a high ratio of viable males relative to females, to minimize the possibility of male limitation constraining fertilisation rates of either con- or heterospecific females in our cultures.

3.3.4.2. Control cultures

Several different controls were required to validate our results: (i) to verify that conspecific mating occurs within the *PbRFP* and *PyGFP* lines, we incubated 75µl of

PbRFP or *PyGFP* infected blood in 5ml culture media; (ii) to check that aphidicolin treatment does not adversely affect *PyGFP* and *PbRFP* females we mixed aphidicolin-incubated parasites with their conspecifics. Specifically, we used *P. berghei Δp47* (only males are fertile) to test *PbRFP* females, and *P. yoelii* 17X wild-type (whose ookinetes are wild-type) to test *PyGFP* females (these cultures followed the same set up as the mixed-species cultures); (iii) to verify that aphidicolin treatment blocked male fertility, we incubated 15µl of *PbRFP* or *PyGFP* infected blood with aphidicolin in 1 ml cultures for 12 min. and washed aphidicolin off as for the mixed-species cultures.

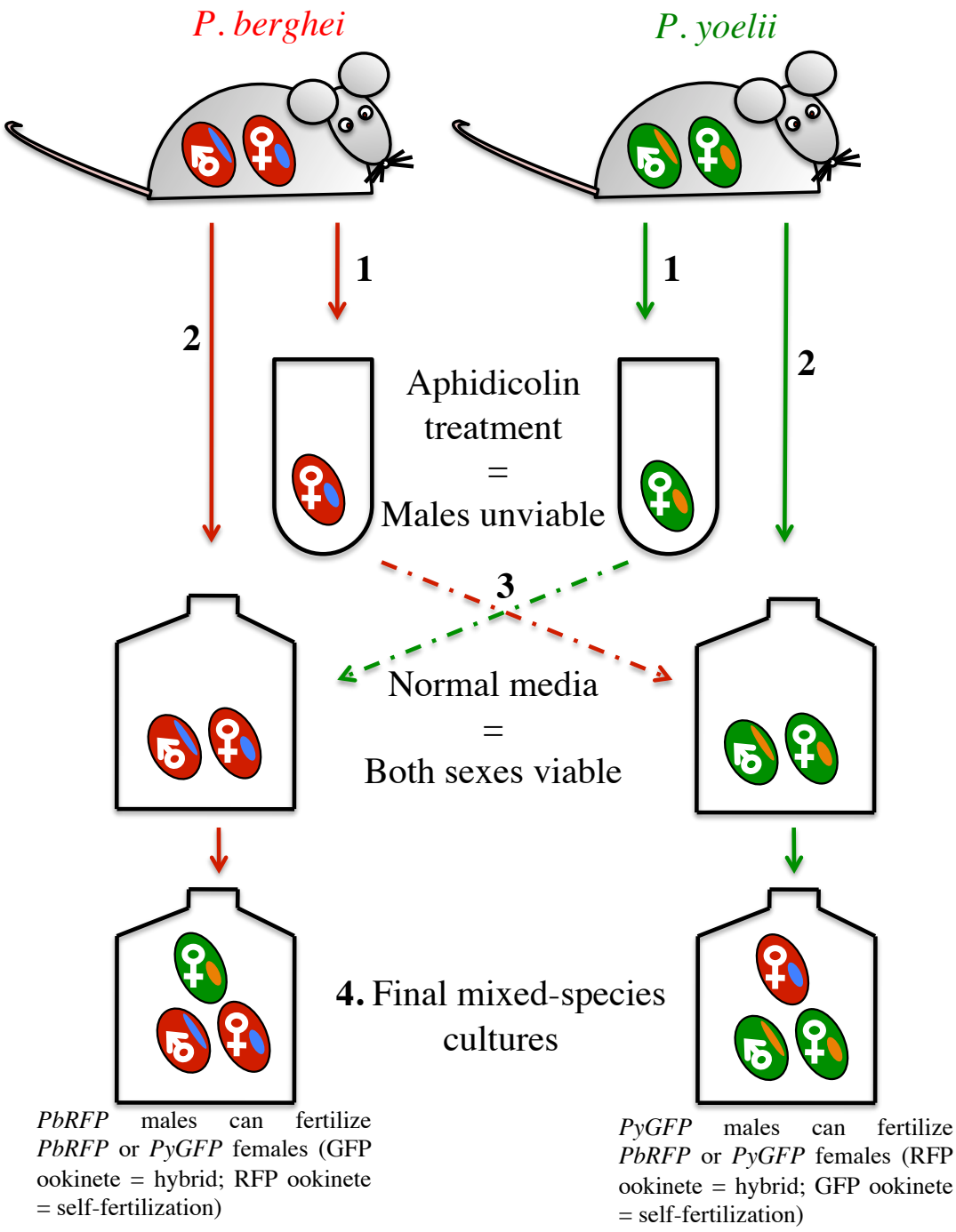

Figure 3.1 Schematic representation for experiment 1.

Blood was taken from mice infected with *P. berghei* (*PbRFP*; red females/ookinetes) or *P. yoelii* (*PyGFP*; green females/ookinetes). (cont.)

Figure 3.1 (cont.) Parasites were incubated in aphidicolin-treated (1) media or not (2). Aphidicolin makes males unviable, leaving only viable females. Aphidicolin was washed and parasites mixed with cultures containing heterospecific parasites (3) in vector mimicking media (i.e. triggers gametogenesis and fertilisation). Final cultures contained males from a single species and females from both species that express different fluorescent proteins (4). In experiment 2, we used KO lines of *P. berghei* whose males are unviable, therefore gametocytes from both species could be directly mixed without aphidicolin treatment. For the full experimental details, please refer to the methods.

3.3.5. Experiment 2: The roles of P230 and P48/45

For experiment 2, we inoculated mice previously treated with 60mg/kg PHZ (day -4 PI), with 10^7 RBCs parasitized with either *P. berghei* wild-type, *PbΔp230*, *PbΔp48/45* or *PyWT*.

3.3.5.1. Mixed-species cultures

We set up crosses between the *P. yoelii* line *PyWT* (unlabelled ookinetes) and one of the *P. berghei* lines *PbΔp230* and *PbΔp48/45* (GFP ookinetes). We mixed 10µl of *PyWT* infected blood (males and females are viable) with 10µl of *PbΔp230* or *PbΔp48/45* (only females are viable) in 1ml cultures. We then assayed the densities of GFP or unlabelled ookinetes. GFP ookinetes result from heterospecific fertilisations between *P. yoelii* males and *P. berghei* females whereas unlabelled ookinetes result from *P. yoelii* self-fertilisations.

3.3.5.2. Control cultures

We set up a series of controls to verify our results: (i) to check that when cultured on their own, *P. berghei* wild-type and *PyWT* produce ookinetes and that *PbΔp230* and *PbΔp48/45* do not, we incubated 20µl of each strain alone in 1ml cultures; (ii) to verify that females from *PbΔp230* and *PbΔp48/45* can be fertilised by conspecific males, we mixed 10µl of *P. berghei* wild-type with 10 µl of *PbΔp230* or *PbΔp48/45* in 1 ml cultures.

Table 3.2 Number of replicate infections for each culture type in experiments 1 and 2.

Controls are numbered for each experiment, following the methods section. For experiment 1, cultures were excluded from analysis if subtracting the number of 'hybrid' ookinetes in mixed-species cultures from the number of ookinetes in control (ii) resulted in negative ookinete counts (see section 3.4.1; results are qualitatively similar if ookinete counts in those cultures are set to 0 and included in the analysis). For experiment 2, cultures were excluded if *PyWT* did not produce ookinetes from conspecific fertilisations in control (i) or in mixed-species cultures.

Experiment	culture type	Line	n (all)	n (analysis)
1	Mixed-species (line hybridizing)	*PyGFP*	22	20
		Pb820	23	15
	control (i)	*Pb820*	24	23
	control (ii)	*PyGFP*	24	23
		Pb820	24	24
	control (iii)	*PyGFP*	24	24
		Pb820	3	3
		PyGFP	3	3
2	Mixed-species	*PbΔp48/45*	20	14
		PbΔp230	17	11
	control (i)	*P. berghei wild-type*	13	13
		PyWT	18	14
	control (ii)	*PbΔp48/45*	18	18
		PbΔp230	15	15
		PbΔp48/45	5	5
		PbΔp230	5	5

3.3.6. Data analysis

All analyses were performed in R v2.14.0 and consisted of generalised linear and linear mixed-effects models, and t-tests, depending on the distribution of the data, the presence of random effects or sample sizes, respectively. Non-parametric Wilcoxon tests were used when assumptions of normality could not be met by data transformation. Most of our analyses involved comparing the level of hybridisation observed to that expected under random mating for each type of culture. We did not simply analyse the densities of hybrid ookinetes observed because the ratio of heterospecific to conspecific females differed across cultures and types of cross (due to *P. berghei* and *P. yoelii* varying in the gametocyte densities they produce). However, since only males from one species are present (in high numbers) in cultures, a useful null-hypothesis arises that, under random mating, the proportion of hybrid ookinetes will not differ from the proportion of heterospecific females. For example, if 25% of the female gametes are heterospecific and if males are randomly mating with the available females, it is expected that 1 hybrid ookinete is produced for every 3 resulting from conspecific mating, such that 25% of the ookinetes will be hybrids. Therefore, hereafter we term the proportion of heterospecific females as the *expected hybridisation* level and the proportion of hybrid ookinetes as the *observed hybridisation* level.

To examine whether density-dependent processes influence hybridisation rates for experiment 2, we used a quasi-binomial generalized linear model to test whether *observed hybridisation* was affected by the following explanatory variables: parasite line, density of con- and hetero-specific females, and their interactions. We minimised models following stepwise deletion of the least significant term and used F-ratio tests (as the model is quasi-binomial) to evaluate the change in model deviance until only significant terms remained (Zuur et al. 2009). Once we obtained the minimal model, we fitted this in a generalized linear mixed-effects framework with infection pair and experimental block as random effects. Additionally, to further examine the relationship between *expected* and *observed hybridisation*, we set up a linear regression between *observed hybridisation* and the *expected hybridisation* (pooling data for the two *P.*

berghei lines) and then used a linear hypothesis test to analyse whether the obtained regression was significantly different from the expected under random mating (i.e. slope = 0 and intercept = 1).

Finally, using the data from experiment 1, we tested whether reproductive interference exists, i.e. whether conspecific ookinete production is reduced in mixed- versus single-species cultures. We first multiplied the number of ookinetes obtained in mixed-species cultures by 1.25 because single-species cultures contained 75 μl of blood but in mixed-species cultures, only 60 μl of the species donating viable male and female gametes was added. We used linear mixed-effects models (with infection pair as a random effect) to test whether the proportion of self-fertilised females was influenced by the presence of heterospecifics. We minimised models using log-likelihood ratio tests.

3.3.7. Molecular evolution

We used molecular evolution tools to investigate the evolutionary patterns and selective pressures shaping the evolution of P230, P48/45, and also P47. We also analyse P47, because the latter is thought to interact with the P230 and P48/45 during fertilisation (van Dijk et al. 2010).

3.3.7.1. PCR and sequencing

We used the DNA samples collected in Chapter 2 to obtain sequence data for P47, P48/45 and P230, from 58 rodent malaria parasite genotypes, covering the four species: *P. berghei*, *P. chabaudi*, *P. vinckei* and *P. yoelii*. As P230 is a large, ~8kb locus, we sequenced two 1kb regions of this locus, which are hereafter referred to as region I and region II. These two regions correspond domains of P230 previously suggested to be fast (region I) or slow (region II) evolving (van Dijk et al. 2010). We designed PCR primers using rodent malaria genome sequences available in PlasmoDB (http://plasmodb.org; Table S3.1) and carried out touch-down PCRs under the following cycling conditions: (i) 95ºC for 3 min.; (ii) 10 cycles of: 94ºC for 30s, 57ºC for 30s (-1ºC per cycle), 72ºC for 1.5 min.; (iii) 35 cycles of: 94ºC for 30s, 47ºC for 30s, 72ºC for 1.5 min.; (iv) 72ºC

for 3 min. If PCR products were negative in gel electrophoresis under these cycling conditions, we carried out new PCR reactions in which the temperatures in step 2 of (ii) and (iii) were increased or decreased by 5ºC. Sequence cleaning, sequencing, assembly, alignment and recombination tests were performed as in Chapter 2. The proportion of genotypes for which we obtained sequence data was always above 86%. For some of the analysis described below, we incorporated sequence data from all the nuclear house-keeping loci described in Chapter 2, the majority of which belong to the same chromosomes as P230 (chromosome 3) or P48/45 and P47 (chromosome 13). We collectively refer to the house-keeping loci as 'control' loci and to P230, P47 and P48/45 as 'mating' loci.

3.3.7.2. Polymorphism, Divergence, Tajima's D and single-locus Mcdonald-Kreitman (MK) tests

We used DNAsp v5 (Librado and Rozas 2009) to compute (i) counts of fixed differences (D_N, D_S) and polymorphisms (P_N, P_S); (ii) divergence ratios (K_A, K_S, K_A/K_S) and nucleotide diversity (π_A, π_S, π_A/π_S) (Nei and Gojobori 1986); (iii) Tajima's D (Tajima 1989); (iv) single-locus MK tests (McDonald and Kreitman 1991). We measured polymorphism for the following groups: *P. chabaudi chabaudi*, *P. vinckei* subsp., *P. yoelii yoelii* (subspecies), *P. chabaudi*, *P. yoelii* (species) and *P. vinckei* subsp.-*P. vinckei petteri*. The latter group was used as these are the only *P. vinckei* subspecies that diverge within the same time frame as the *P. chabaudi* and *P. yoelii* subspecies (others are much older; see Chapter 2). We estimated divergence from an inferred ancestral sequence (as in Chapter 2), for the *P. berghei-P. yoelii* and *P. chabaudi-P. vinckei* nodes. We did not use *P. berghei* as an intraspecific group due to the previously shown lack of polymorphism.

3.3.7.3. Maximum-likelihood MK and Hudson-Kreitman-Aguade (HKA) tests

We used maximum likelihood versions of both MK and HKA tests published in (Welch 2006) and (Wright and Charlesworth 2004), respectively. These extend the original tests

to allow for the analysis of multi-locus data and for the comparison of selective patterns in different classes of loci, thus allowing us to compare mating and control loci. We computed these methods using the polymorphism and divergence estimates described in the section above and used data for all control loci and either all, or each of, the mating loci separately.

The MK test infers positive selection if the number of non-synonymous fixed differences (D_N) is larger than what is expected under the neutral model. The data used to compute MK tests (D_N, D_S, P_N, P_S) can also be used to estimate α, which is the proportion of non-synonymous substitutions due to positive selection (Eyre-walker 2006). For this test, we used three models in which: (i) α is constrained to zero at all loci, i.e. no adaptive evolution; (ii) α is a free-parameter common to all loci; or (iii) α is free to differ between mating and control loci. This allows us to test whether positive selection exists and if there is a difference between mating and control loci. We compared models using AICc and calculated Akaike weights following (Burnham and Anderson 2002; Akaike weights represent the probability that a model is the best in a given model set). We obtained estimates of α under model (iii) for mating and control loci.

The HKA test detects unusually high or low levels of diversity, given the observed level of divergence between different taxa. This can be used to infer the action of balancing selection where this has resulted in elevated diversity, or selective sweeps where this has resulted in a local decrease in genetic diversity (Kreitman 2000). We compared models in which there is no selection at both 'mating' and 'control' loci to models in which selection is allowed to occur at 'mating' loci, and significance was assessed using likelihood ratio tests.

3.3.7.4. The identification of codons evolving under position selection

To test for variation in the strength of selection along a locus, we fitted two sets of nested codon evolution models (with PAML; Yang 2007) to the data for each mating loci. The different models either constrain all codons to be evolving at a neutral or constrained ratio of non-synonymous to synonymous divergence ($K_A/K_S \leq 1$; M1a, M7 and M8a) or allow for a proportion of codons to be evolving under positive selection ($K_A/K_S > 1$; M2a and M8). We compared models using likelihood ratio tests between M8 and M7 (or M8a) and between M2a and M1a. When M2a or M8 fitted the data significantly better, indicating the presence of positive selection, we used a Bayes Empirical Bayes (BEB) approach to identify specific codons affected.

3.4. RESULTS

3.4.1. Experiment 1: reproductive barriers

We obtained hybrid ookinetes in crosses between male *PbRFP* and female *PyGFP* (in 40% of the cultures), and between male *PyGFP* and female *PbRFP* (in 60% of the cultures). However, the proportion of hybrid ookinetes *(observed hybridisation*) was much lower than the proportion of heterospecific females (*expected hybridisation*; Figures 3.2A-B). Specifically, in the cross between *PbRFP* males and *PyGFP* females, *expected hybridisation* was on average 0.16 ± 0.03 (± standard error) but *observed hybridisation* was 0.03 ± 0.01 ($V = 0$; $df = 19$; $p < 0.0001$), and in the cross between *PyGFP* males and *PbRFP* females, *expected hybridisation* was 0.85 ± 0.02 and *observed hybridisation* was 0.15 ± 0.06 ($V = 5$; $p < 0.0001$; $df = 14$).

These findings are validated by our control cultures in which: (i) conspecific matings occur readily in *PbRFP* and *PyGFP* and there is no significant difference between the proportion of fertilised females for either species (Likelihood ratio test [LRT]: $\chi^2_1 = 0.448$; $p = 0.503$); (ii) aphidicolin treatment does not affect the fertility of *PyGFP* or *PbRFP* females as there is no significant difference between the proportion of fertilised females with and without aphidicolin for either species (*PbRFP*: $t = 3.63$, $df = 2$, $p =$

0.0682; *PyGFP*: t = 2.208, df = 2, p = 0.158); (iii) aphidicolin affects males and on average, reduced ookinete production by 99.4% for *PbRFP* (95% confidence interval [CI]: 99.0 – 99.8) and by 95.7% for *PyGFP* (CI: 90.2 – 100). The low rate of males escaping aphidicolin did not result in a significant difference between the proportion of fertilised females for each parasite species (LRT χ^2_1 = 2.12; p = 0.146). Therefore, for the mixed-species cultures (i.e. in the experimental results presented in the paragraph above), we corrected for the potential presence of aphidicolin escapes by subtracting the number of ookinetes/ml produced by escaped males in the aphidicolin controls from the density of hybrid ookinetes observed. Because each pair of infections contributed to each experimental and control treatment we were able to apply the specific correction value for each infection pair. Given that hybridisation was still observed after performing this correction, the observed hybrid ookinetes are unlikely to be due to males that escaped aphidicolin treatment.

3.4.2. Experiment 2: the roles of P230 and P48/45

In this experiment, we obtained hybrids between male *PyWT* and females from both *PbΔp230* (~80% of the cultures) and *PbΔp48/45* (~90% of the cultures). In contrast to experiment 1, *observed hybridisation* was much higher for experiment 2 and approached expected levels under the assumption of random mating (Figures 3.2C-D). Specifically, whilst the mean difference between *expected* and *observed hybridisation* was 0.70 (95% CI: 0.58-0.83; assuming a normal distribution) for crosses between *PyGFP* males and *PbRFP* females (experiment 1), this was reduced to 0.14 (95% CI: 0.016-0.28) and 0.16 (95% CI: -0.04-0.36) for crosses between *PyWT* males and *PbΔp230* or *PbΔp48/45* females, respectively. Moreover, there was a significant difference between *expected* (0.71 ± 0.07) and *observed hybridisation* (0.56 ± 0.09) for *PbΔp230* (t = -2.49, df = 10, p = 0.032), but this was not the case for *PbΔp48/45* (t = -1.75, df = 13, p = 0.104; *observed hybridisation*: 0.39 ± 0.10), suggesting that *PyWT* males randomly mated with conspecific and *PbΔp48/45* females. These results suggest that P230 and P48/45 play a major role in pre-zygotic reproductive barriers between species.

As in the previous experiment, the control cultures validate our results: (i) when cultured alone *PbΔp230* and *PbΔp48/45* cultures did not produce ookinetes but *P. berghei* wild-type and *PyWT* did, and there was no significant difference between the proportion of fertilised females for each species ($W = 86.5$, $p = 0.8461$); (ii) *PbΔp230* and *PbΔp48/45* females can be fertilised by *P. berghei* wild-type males and the latter show no preference for either wild-type females or females from *PbΔp230* ($t = 1.2578$, $df = 4$, $p = 0.2769$) or *PbΔp48/45* ($t = -1.2291$, $df = 4$, $p = 0.2864$).

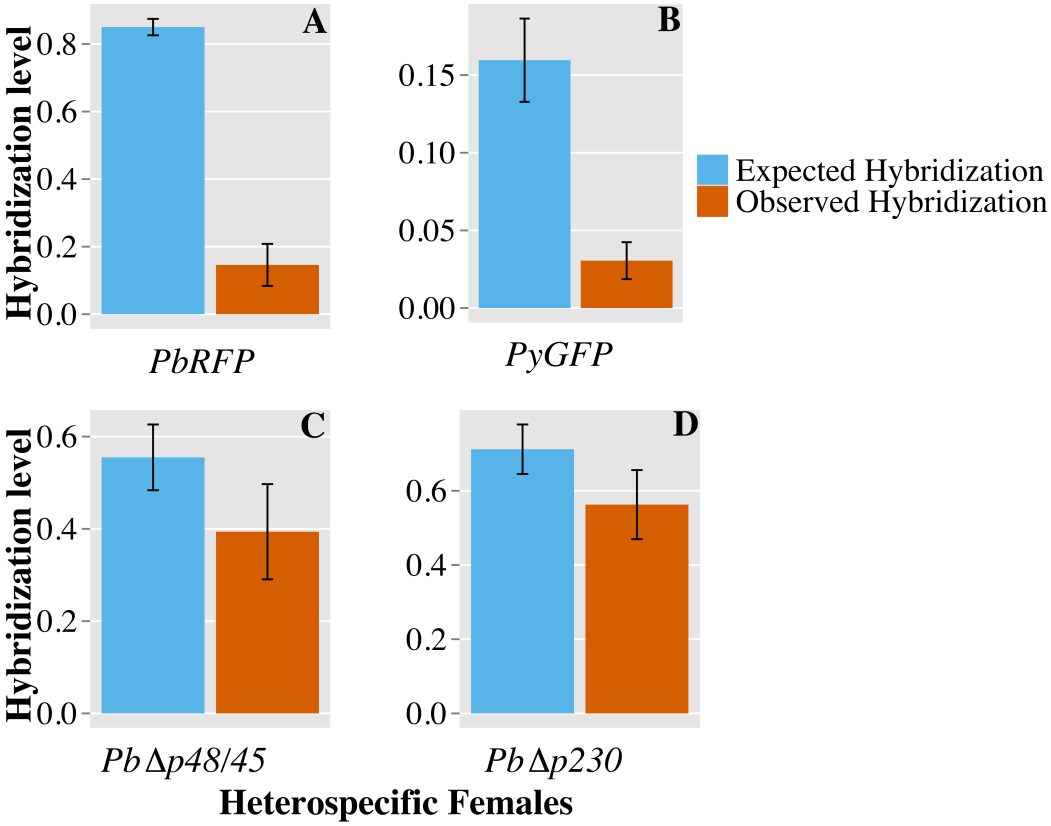

Figure 3.2 Hybridisation occurs between *P. berghei* and *P. yoelii*, especially in the absence of P230 and P48/45.

Levels of *expected* (under random mating) and *observed hybridisation* for 'wild-type' (A and B; experiment 1) and KO parasite lines (C and D; experiment 2). Similar levels of *expected* and *observed hybridisation* suggest low discrimination between con- and heterospecific mates. x-axis labels represent the strain (cont.)

Figure 3.2 (cont.) donating heterospecific females into culture. The crosses shown are between *PyGFP* males and *PbRFP females* (A), *PbRFP* males and *PyGFP* females (B), *PyWT* males and *PbΔp48/45* (C), *PyWT* males and *PbΔp230* (D).

3.4.3. Hybridisation and non-random mating

The results from Experiment 2 suggested that *P. yoelii* males could randomly mate with conspecific or *PbΔp48/45* females. We next used our data to further examine this and investigate whether density-dependent processes affect mating rates. We tested whether the level of *observed hybridisation* was affected by the density of con- and heterospecific females and by the identity of the *P. berghei* line (*PbΔp230* or *PbΔp48/45*). The identity of the *P. berghei* line does not have a significant effect on *observed hybridisation* ($F_{(1,20)} = 0.813$; $p = 0.378$), but this is significantly affected by the interaction between the densities of con- and heterospecific females in culture ($F_{(1,21)} = 10.199$, $p = 0.004$). This interaction can be visualized in Figure 3.3. The level of *observed hybridisation* was relatively low across most of the parameter space, but generally increases with the density of heterospecific females and decreases with the density of conspecific females. However, these changes are non-linear, especially at intermediate densities of heterospecific females. Specifically, when the density of heterospecific females is low, the level of *observed hybridisation* is dominated by the density of conspecific females (colours change horizontally). On the other hand, at high densities of heterospecific females (>100x10^6/ml), conspecific females are overwhelmed and the level of *observed hybridisation* becomes independent of the density of conspecific females (colours change vertically). Moreover, the parameter space producing random mating is highly constrained, occurring only at very low (<20x10^6/ml) or very high (>140x10^6/ml) densities of heterospecific females. Finally, as there was no significant effect of *P. berghei* line, we pooled the lines and fitted a regression between *observed* and *expected hybridisation*, obtaining a slope of 0.86 and an intercept of -0.07. This was significantly different from what would be expected

under random mating (slope = 1, intercept = 0; $F_{(2,23)} = 3.85$; $p = 0.036$; linear hypothesis test), suggesting that mating is non-random. Overall, these results indicate that mating is non-random and that there are complex interactions between hybridisation and the density of con- and heteropsecific females available for fertilisation.

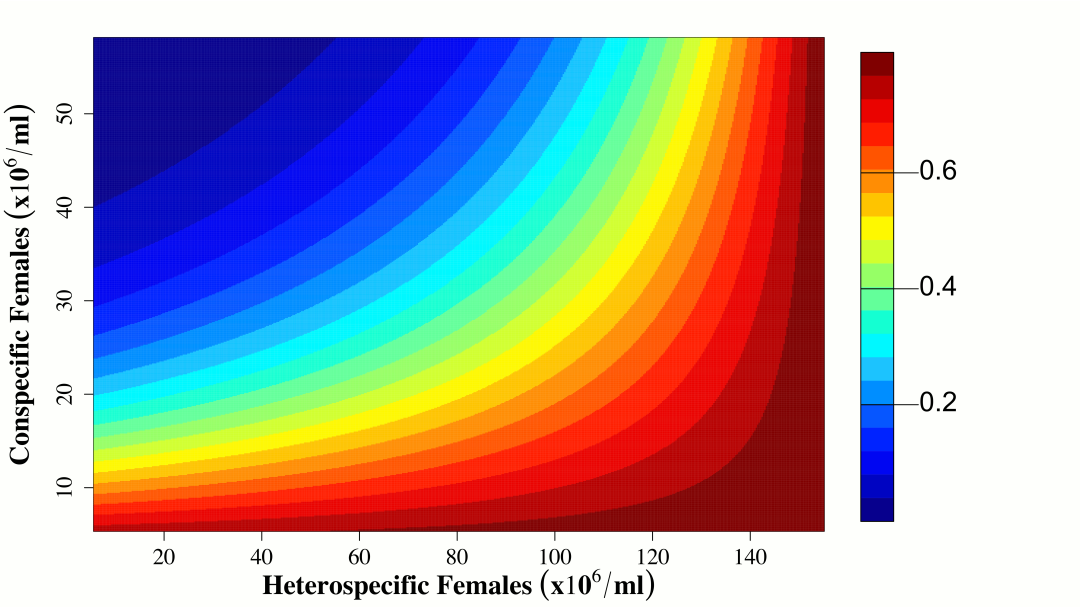

Figure 3.3 The level of *observed hybridisation* is influenced by an interaction between the density of con- (*P. yoelii*) and heterospecific (*P. berghei Δp230* or *Δp48/45*) females.

The level *of observed hybridisation* (colour scale) is affected, in a non-linear manner, by the interaction between con- and heterospeciffic females. (see '*Hybridisation and non-random mating'* in the 'Results' section for further explanation). Additionally, *observed hybridisation* levels are normally lower than expected under random mating for any given combination of con- and heterospecific female densities. For example, imagine we had $20x10^6$ conspecific females and the same density of heterospecific females. This would make the proportion of heterospecific females in culture (*expected hybridisation*) equal to 0.5, which matches yellow on the scale bar. However, when the density of con- and heterospecific females is $20x10^6$, the obtained colour is cyan, (cont.)

Figure 3.3 (cont.) which corresponds to an *observed hybridisation* of ~0.3 (20% lower than what would be expected under random mating).

3.4.4. Reproductive interference

Finally, to further explore the ecology of mating interactions between different *Plasmodium* species, we tested whether the presence of heterospecific females in mixed-species cultures had an effect on the conspecific fertilisation level (i.e. reproductive interference). Here, we used data collected for experiment 1 and show that for *P. berghei* the rate of self-fertilisation is significantly affected by the presence of *P. yoelii* (LRT $\chi^2_4 = 18.95$, $p < 0.001$, single-species cultures: 0.31 ± 0.03; mixed-species: 0.20 ± 0.03). In contrast, the presence of *P. berghei* had no effect on the rate of *P. yoelii* self-fertilisation (LRT $\chi^2_6 = 5.26$; $p = 0.51$, single species: 0.28 ± 0.05). These results demonstrate asymmetric reproductive interference between these parasite species (Figure 3.4).

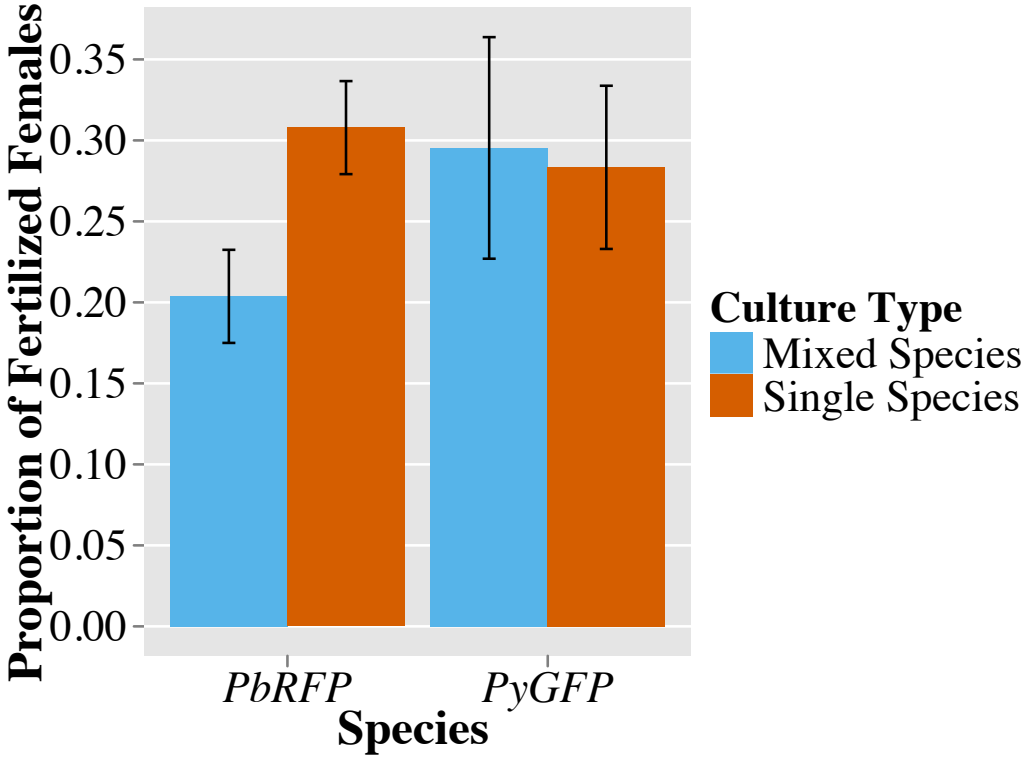

Figure 3.4 Asymmetric reproductive interference.

Proportion of self-fertilised females in single- and mixed-species cultures, for *PbRFP* and *PyGFP*. If the presence of heterospecifics had no impact on fertilisation success, the proportion of self-fertilised females would not significantly differ between mixed- and single-species cultures. This is the case for *PyGFP*, but fertilisation success is significantly reduced for *PbRFP* in mixed-species cultures.

3.4.5. Molecular evolution

3.4.5.1. Polymorphism, Divergence, Tajima's D, MK and HKA tests

Average polymorphism (π_S) was ~0.01 and average divergence (K_A/K_S) varied between 0.3 and 0.5 (depending on the species/subspecies being compared; Table S3.2). The single gene MK tests were significant only for region I of P230, with α varying between 0.7 and 0.85 (Table S3.2). Accordingly, the multi-locus MK tests show that the mating

loci are evolving significantly faster than the control loci and that this is mostly driven by region I of P230 and P47 (i.e. Akaike weights show that model (iii) provided a better fit to the data). From model (iii), we obtained α, which varied between 0.7-0.85 and 0.49-0.82 for P230 region I and P47, respectively. We obtain similar results for the multi-locus MK test at the subspecies (Table 3.3) and the species level (Table S3.3). On the other hand, the HKA test did not detect differences in diversity between mating and control loci, for any of the comparisons tested, suggesting an absence either of long-term balancing selection or recent selective sweeps (Kreitman 2000). In agreement, single locus Tajima's D were always non-significant. This suggests that the mating genes (particularly P47 and region I of P230) are evolving under positive selection, but we observe no evidence for balancing selection or recent selective sweeps.

Table 3.3 Multi-locus MK tests for the subspecies.

This analysis was carried using all control loci and either all or each of the mating loci separately. The Akaike weight for model (iii) (allows *α* to vary between mating and control loci) is given and highlighted in bold when this is the best model. Akaike weights are calculated for the set of models tested and always add up to 1. *α*, obtained under model (iii) is given for the mating and the control loci. For a given subspecies, the values of *α* for the control loci change with the mating locus used, even though the data for the control loci was the same. This is because the likelihood models include the following parameters that we assume to be constant across all loci: neutral diversity and divergence per site, and selective constraint.

locus	class	*P. c. chabaudi*		*P. v.*. subsp.		*P. y. yoelii*	
		Akaike weight	**α**	**Akaike weight**	**α**	**Akaike weight**	**α**
all	mating	**0.99**	0.75	**0.99**	0.63	**0.99**	0.46
	control		0.37		0.006		-0.94
P230 region I	mating	**0.99**	0.86	**0.99**	0.81	**0.99**	0.71
	control		0.47		0.50		-0.55
P230 region II	mating	0.29	0.67	0.28	0.56	0.14	-0.89
	control		0.47		-0.02		-0.98
P47	mating	**0.99**	0.84	**0.76**	0.66	**0.99**	0.73
	control		0.39		0.08		-0.51
P48/45	mating	0.15	0.45	0.48	0.67	0.18	0.20
	control		0.47		0.26		-0.42

3.4.5.2. Codon evolution and variation in selection among codons

As shown in Table 3.4, codon evolution models detect positive selection across all mating loci, with most model comparisons being significant. The estimated K_A/K_S for the class of codons under positive selection was always above 2.3 and whilst the percentage of codons in this class varied between loci and codon evolution model, it was the highest for P47, followed by P230 region I, P48/45 and finally P230 region II. Interestingly, as can be seen in Table 3.5, our analysis suggests that all but two of the codons identified in (van Dijk et al. 2010) are under positive selection and greatly expands their list. However, only a small number of codons show high support for positive selection (posterior probability >0.9). Thus, whilst we detect positive selection in the mating loci, the specific identity of the fast evolving codons should be treated with care.

Table 3.4 Codon substitution models and likelihood ratio tests.

Likelihood ratio tests comparing nested models of codon evolution and estimates of the value of K_A/K_S and the % codons under positive selection (K_A/K_S >1) for each locus. M1a, M7 and M8a assume neutral evolution (K_A/K_S ≤1) and models M2a and M8 assume there is a class of codons under positive (K_A/K_S >1). $p < 0.05$ indicate that positive selection was detected.

locus	model comparison	likelihood ratios and *p*-values	no. of codons analyzed	% selected codons and K_A/K_S of selected class for M2a or M8
P230 region I	M1a vs. M2a	χ^2_2= 0; p=1	409	M2a: -
	M7 vs. M8	χ^2_2=13.11; p=0.0014		M8: 3.5%; K_A/K_S=2.38
	M8a vs. M8	χ^2_1=3.16; p=0.038		
P230 region II	M1a vs. M2a	χ^2_2=0; p=1	365	M2a: -
	M7 vs. M8	χ^2_2=10.62; p=0.005		M8: 1.5%; K_A/K_S=3.83
	M8a vs. M8	χ^2_1=5.79; p=0.008		
P47	M1a vs. M2a	χ^2_2=8.65; p=0.013	428	M2a: 5.9%; K_A/K_S=2.71
	M7 vs. M8	χ^2_2=13.67; p=0.001		M8: 9.3%; K_A/K_S=2.37
	M8a vs. M8	χ^2_1= 9.74; p=0.001		
P48/45	M1a vs. M2a	χ^2_2=4.58; p=0.10	478	M2a: 1.9%; K_A/K_S=3.67
	M7 vs. M8	χ^2_2=9.04; p=0.011		M8: 3.1%; K_A/K_S=3.17
	M8a vs. M8	χ^2_1=5.44; p=0.01		

Table 3.5 Codons with $K_A/K_S > 1$, as identified by Bayes Empirical Bayes (BEB) analysis.

Codons in bold were detected by BEB in both M8 and M2a. Labelled codons have a posterior probability $p > 0.90$ (#) or $p > 0.95$ (*) or have been previously suggested to be under positive selection (§) by (van Dijk et al. 2010). Codons in which ? replaces the single letter amino acid code correspond to missing data in the sequence used by PAML as reference for codon identity.

locus	codons with $K_A/K_S>1$
P230 region I	70T, 92F, 95S, 98V(§), 113D(#), 144N(#), 167Q, 171V, 202S, 254N, 340S, 381?
P230 region II	25?(#), 27?, 82L, 89V, 127L(#), 139L, 173K, 259L, 336?(#)
P47	**9?(§)**, 12?, 27F(§), **32V(§)**, 69I, 71L, 79N(§), 82E(§), 84M, 125S, 155R(§), **158I(*)**, 162G, **163E(§)**, **165I(§)**, 182Q, 186Q(§), **195T(*)**, 213V, 219G, 243L, 275K, 277K, 289V, **293K**, **324A**, 350N, 354V, 387I, **400H**, **404K**, 428?
P48/45	**12?, 30?, 111I, 139K, 140T(#, §), 164S, 204T, 220Q, 230N, 233D(§), 361S(*, §), 416I**

3.5. DISCUSSION

We combined *in vitro* fertilisation experiments with molecular evolution tools to study the biology of fertilisation in malaria parasites. First, our experiments demonstrate that hybridisation can occur (at low levels) between different species of rodent malaria parasites. Second, heterospecific matings occur at high levels in the absence of either one of two transmission-blocking targets (P230 or P48/45) on the surface of females. Third, we show that mating between females without P230 or P48/45 and heterospecific

males is non-random, suggesting that other factors are also involved in gamete recognition. Fourth, we show the existence of reproductive interference in which mating between con-specifics is reduced by the presence of another species. Fifth, we show that P230, P48/45, and the potential interaction partner P47, are under strong positive selection. Below, we discuss the roles of P230 and P48/45 in mating and the implications of our results for the development of interventions to control malaria.

3.5.1. The role of P230 and P48/45 in recognition and attachment: biological implications and possible molecular interactions

Whilst the protein composition of the gamete surface is not entirely known, P230 and P48/45 form a complex (anchored to the membrane by P48/45) at the surface of gametes (Kumar 1987; Eksi et al. 2006). Thus, the deletion of P48/45 also results in the loss of P230 (Eksi et al. 2006), explaining why we observe the same phenotypes for both *PbΔp230* and *PbΔp48/45*. However, whilst P230 is expressed at the surface of *P. falciparum* female gametes, for *P. berghei* this has not been conclusively shown, but if P230 was not expressed on *P. berghei* females, we would expect the *PbΔp230* female phenotype to be the same as wild-type. Therefore, our results suggest that females require an intact P230-P48/45 complex to prevent heterospecific mating (Figure 3.2). Combing our finding with previous studies (summarized in Figure 3.5) showing that the presence of P230 and P48/45 at the surface of male gametes is essential for males to attach to females (van Dijk et al. 2010), allows us to form the following conclusions and testable predictions.

(1) P230-P48/45 complex has sex-specific roles in attachment (males) and recognition (females). These roles could be simply mediated by differences in the protein composition of male and female gamete surfaces, due to variation in expression levels or differences on the specific proteins expressed. Such differences would affect the supramolecular structure of the gamete surface, which can be important for attachment/recognition (Hoodbhoy and Dean 2004). Additionally, in *P. falciparum*, P230 is cleaved during gametogenesis resulting in 4 fragments: two large fragments

(~300 KDa) that remain at the surface of gametes and two small peptides that may be released into the blood meal (Brooks and Williamson 2000). If the cleavage status of P230 is sex-specific, this could also produce sex-specific roles.

(2) Recognition is non-essential for fertilisation. Whilst this may seem surprising, it is also observed in mammals, where the removal of the zona pellucida proteins (which surround female gametes and mediate recognition) allows gametes from organisms as divergent as humans and hamsters to achieve fertilisation (Wassarman et al. 2001).

(3) Attachment and recognition may be independent processes in malaria parasites. Whether this is the case will depend on whether the ligands involved in those processes are shared. Given that P230 and P48/45 are involved in attachment in males and recognition in females, attachment and recognition will only use the same set of ligands if the P230-P48/45 complexes at the female and male surfaces directly interact. Although, this hypothesis is possible for recognition, we suggest that it is less likely for attachment. As shown in Figure 3.5, recognition depends on the female P230-P48/45 complex, but attachment only occurs if this complex is expressed by males (van Dijk et al. 2010). Thus, recognition requires intact P230-P48/45 at the surface of both sexes and could be mediated by interactions between them. Conversely, P230-P48/45 at the surface of males mediates attachment independently of the expression of this complex at the surface of females. Therefore, if male-female P230-P48/45 complexes directly mediate attachment, male gametes would have to switch to a different attachment ligand when this complex is absent from the female surface. We suggest that a more parsimonious explanation is that male P230-P48/45 normally attaches to a different set of female ligands (e.g. P47).

(4) Despite the absence of an intact P230-P48/45 complex from the surface of heterospecific females, *P. yoelii* males still showed some preference for fertilizing conspecific females, suggesting that additional factors mediate recognition/attachment (Figures 3.3). This could involve species-specific chemotatic signals or other protein-protein interactions at the surface of male and female gametes. Chemotaxis is involved in the migration of sporozoites (Akaki and Dvorak 2005), but is yet to be investigated for gametes. Additionally, recognition could also be partially mediated by interactions at

the gamete surface with proteins such as the LAP/CCp family (which are non-essential for fertilisation; Raine et al. 2007) or P47.

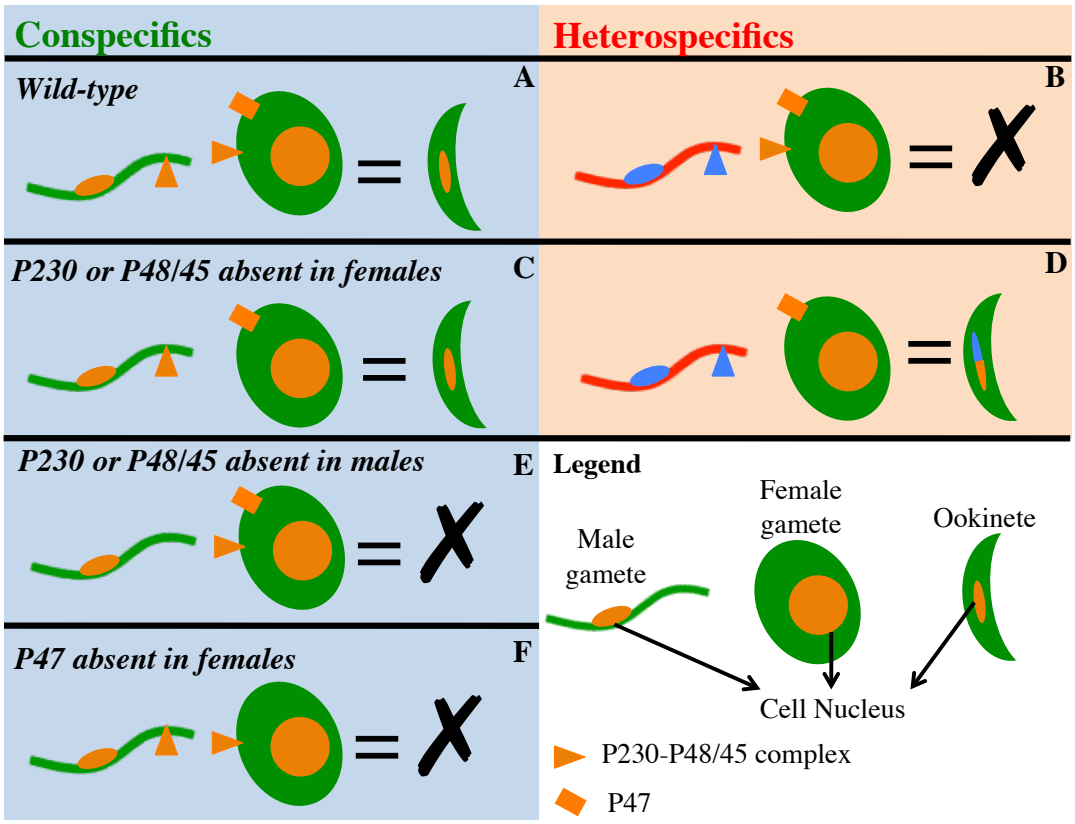

Figure 3.5 Summary of the effects of the absence of P230, P47 or P48/45 from the surface of male or female gametes.

The conspecifics column refers to experiments with *P. berghei* and the heterospecifics column refers to crosses between *P. yoelii* males and *P. berghei* females, as described in this chapter. Ookinetes are produced at a high rate when mating occurs between wild type conspecifics (A), but not wild type heterospecifics (B). In the absence of P230 or P48/45 from the female surface (P230-P48/45 complex disrupted), both con- (C) (van Dijk et al. 2001; van Dijk et al. 2010) and heterospecific crosses (D) readily produce ookinetes. In conspecific crosses, the absence of P230 or P48/45 from the surface of male gametes (E) or the absence of P47 from the surface (cont.)

Figure 3.5 (cont.) of female gametes (F) prevents ookinete formation (van Dijk et al. 2001; van Dijk et al. 2010). *P. berghei* gametes and ookinetes resulting from conspecific fertilisations are coloured with a green cytoplasm, orange nucleus and gamete surface proteins. *P. yoelii* gametes are coloured in red, with blue nucleus and gamete surface proteins. Hybrid ookinetes (D) are coloured with a green cytoplasm (same as the female involved in the cross) and a blue and orange nucleus, representing the genetic material from two species.

3.5.2. Hybridisation and introgression

Our results showed that hybridisation between 'wild-type' malaria parasites occurred at much lower levels than conspecific matings, but was clearly non-zero (Experiment 1; Figure 3.2A-B). Thus, an important question is whether hybrids can complete the life-cycle. To begin to study this, we carried out a small number of mosquito feeds on mice co-infected with *P. yoelii* and *P. berghei* GFP lacking P230 or P48/45. We observed hybrid oocysts (GFP) of abnormal size (without sporozoites) in mixed, but not in single-species infections (data not shown). This suggests that although hybrids may fail to proceed further than forming oocysts, the nuclei of *P. yoelii* males and *P. berghei* females may recombine (this has been suggested to occur 2-3h after fertilisation, but has not been conclusively demonstrated). The viability of hybrids deserves further attention, because even if hybrid parasites are only rarely viable, introgression can have an important role in evolution (Huyse et al. 2009; Rieseberg 2009; Song et al. 2011). For example, introgression has led to the acquisition of pesticide resistance by the European house mouse and enabled host-range expansion in schistosomes (Huyse et al. 2009; Song et al. 2011). Similar situations in malaria parasites (e.g. the acquisition of drug resistance) could strongly influence disease dynamics and control efforts. Additionally, hybridisation often plays an important role in the establishment of invasive species or in the adaptation to novel environments (Lee 2002; Huyse et al. 2009; Senn et al. 2009) and could therefore be particularly important for parasite adaptation during a host-switch event. Importantly, such an event may be currently occurring with *P. knowlesi*, a

macaque parasite that has recently been shown to infect humans (Singh et al. 2004). However, demonstrating hybridisation/introgression in the wild can be difficult, as it requires the identification of incongruences between gene trees from different loci, whilst eliminating other sources for the same effect, as incomplete lineage sorting or gene duplication (Joly et al. 2009). Nevertheless, evidence for hybridisation can be obtained if strong incongruences are detected between mitochondrial and nuclear loci sequenced from single genotypes, as in the case of schistossomes (Huyse et al. 2009). For malaria parasites, these data could be obtained from the wild by extracting DNA from single oocysts (which contain thousands of parasite genomes) (Anthony et al. 2007) or by cloning single genotypes from wild isolates (Nkhoma et al. 2012).

3.5.3. Molecular evolution of P230, P48/45 and P47

In other organisms, gamete recognition proteins are fast evolving and this is thought to be driven by sexual conflict/selection (e.g. polyspermy, assortative mating) or reinforcement (Swanson and Vacquier 2002; Geyer and Palumbi 2003; Palumbi 2009; Levitan and Stapper 2010). Our results also show strong evidence of adaptive evolution of P230, P48/45, and P47 (mating loci). Specifically, we find that: (i) models allowing for positive selection fit the data significantly better than neutral models at all loci; and (ii) MK tests suggest that the mating loci are evolving faster than a set of house-keeping (control) loci and that this is mainly driven by region I of P230 and P47. Furthermore, our analyses identified several fast evolving codons for the mating loci. Eight of these codons belong to domain IV of P230 as defined in (Gerloff et al. 2005). The 3D structure of *P. falciparum* P230 indicates that this domain is available for molecular interactions, suggesting it could be a candidate for mediating gamete recognition. Domain IV is also relevant because several non-synonymous polymorphisms have been identified in *P. falciparum* and it has been recently included in a *P. vivax* vaccine formulation (Gerloff et al. 2005; Tachibana et al. 2012).

Whilst we detect positive selection on the mating loci, we can mostly speculate about the ecological factors driving this. In malaria, an important selective pressure is the immune response, because P230 and P48/45 are important triggers of natural antibody responses that can reduce fertilisation and transmission (Healer et al. 1999; Bousema et al. 2010). If this is the case, P230 and P48/45 would both be under divergent selection (due to immunity) and contributing towards non-random mating. Such a pleiotropic effect cannot be broken by recombination and can facilitate the process of speciation, particularly in the presence of gene flow (Servedio et al. 2011). This implies that immunity against P230 or P48/45 could accelerate the rate at which *Plasmodium* speciates. In contrast, no immune responses have been detected against P47 (van Schaijk et al. 2006), suggesting that other forces are the main drivers of the evolution of this protein.

3.5.4. Reproductive interference

We detected asymmetric reproductive interference (Figure 3.4), i.e. the self-fertilisation success of *P. berghei*, but not *P. yoelii*, is reduced by a third in the presence of heterospecific females. Such an effect could be mediated by: (i) immunity, (ii) chemotaxis, or (iii) allelopathy, providing that *P. berghei* and *P. yoelii* vary in their production or response to these factors (e.g. *P. yoelii* males can distinguish between con- and heterospecific chemotatic signals, but *P. berghei* males can not). (Paul et al. 2002) showed that reproductive interference also occurs among avian malaria parasites and that this is independent of immunity, suggesting that direct interactions among parasites are involved. An important consequence of asymmetric reproductive interference is that one species (in our case, *P. berghei*) will suffer reduced transmission when in co-infections. Therefore, reproductive interference may be important in determining the epidemiological dynamics and the geographic distribution of malaria parasites.

3.5.5. Transmission-blocking vaccines

Whilst the functional role of P230 and P48/45 is in gametes, these proteins are also expressed in gametocytes circulating in the blood and trigger antibody responses that greatly reduce transmission (Healer et al. 1999; Bousema et al. 2010). Consequently, both proteins are being actively studied as possible targets for transmission blocking vaccines (Bustamante et al. 2000; Outchkourov et al. 2008; Chowdhury et al. 2009; Doi et al. 2011; Tachibana et al. 2012). Antibodies against P230 and P48/45 can block fertilisation by two main mechanisms: (i) complement mediated lysis of gametes; or (ii) preventing gamete attachment (Carter et al. 1990; Healer et al. 1997; Sutherland 2009; Tachibana et al. 2012). Whilst the former should prevent male-female interactions, the latter will not. Given that vaccines will tend to be species-specific, our results suggest vaccination may allow females of the target species to be fertilised by heterospecific males in mixed-species infections. On the other hand, if as suggested above, immune pressure drives the evolution of recognition proteins, vaccines targeting fast evolving epitopes of P230 or P48/45 could have additional benefits if they increase the rate at which populations subdivide, because by making mating and gene flow more local, the spread of vaccine escape mutants and drug resistance will be constrained. Clearly further work is required to determine whether targeting P230 and P48/45 will have favourable or unfavourable evolutionary consequences.

3.6. APPENDIX

Table S3.1. Primers used to sequence P230, P47 and P48/45 (Electronic version only).

Table S3.2. Polymorphism, divergence and single locus MK tests and Tajima's D (silent sites) (Electronic version only).

Table S3.3 Multi-locus MK tests for the species.

Table S3.3 Multi-locus MK tests for the species. This analysis was carried using all control loci and either all or each of the mating loci separately. The Akaike weight for model (iii) (allows α to vary between mating and control loci) is given and highlighted in bold when this is the best model. Akaike weights are calculated for the set of models tested and always add up to 1. α, obtained under model (iii) is given for the mating and the control loci. For a given subspecies, the values of α for the control loci change with the mating locus used, even though the data for the control loci was the same. This is because the likelihood models include the following parameters that we assume to be constant across all loci: neutral diversity and divergence per site, and selective constraint.

locus	**class**	***P. chabaudi***		***P. v.* subsp. – *P. v. petteri***		***P. yoelii***	
		Akaike weight	**α**	**Akaike weight**	**α**	**Akaike weight**	**α**
all	**mating**	**0.98**	**0.66**	**0.99**	**0.71**	**0.99**	**0.42**
	control		**0.31**		**0.14**		**-1.35**
P230 region I	**mating**	**0.99**	**0.86**	**0.99**	**0.85**	**0.99**	**0.75**
	control		**0.5**		**0.37**		**-0.62**
P230 region II	mating	0.3	0.68	0.45	0.58	0.14	-0.45
	Control		0.52		0.2		-0.54
P47	**mating**	**0.98**	**0.74**	**0.9**	**0.74**	**0.99**	**0.52**
	control		**0.29**		**0.21**		**-1.53**
P48/45	mating	0.21	0.4	0.68	0.74	0.42	0.31
	control		0.54		0.38		-0.58

4. Sex and Death: the Effects of Innate Immune Factors on the Sexual Reproduction of Malaria Parasites

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4.1. SUMMARY

Malaria parasites must undergo a round of sexual reproduction in the blood meal of a mosquito vector to be transmitted between hosts. Developing a transmission-blocking intervention to prevent parasites from mating is a major goal of biomedicine, but its effectiveness could be compromised if parasites can compensate by simply adjusting their sex allocation strategies. Recently, the application of evolutionary theory for sex allocation has been supported by experiments demonstrating that malaria parasites adjust their sex ratios in response to infection genetic diversity, precisely as predicted. Theory also predicts that parasites should adjust sex allocation in response to host immunity. Whilst data are supportive, the assumptions underlying this prediction – that host immune responses have differential effects on the mating ability of males and females – have not yet been tested. Here, we combine experimental work with theoretical models in order to investigate whether the development and fertility of male and female parasites is affected by innate immune factors and develop new theory to predict how parasites' sex allocation strategies should evolve in response to the observed effects. Specifically, we demonstrate that reactive nitrogen species impair gametogenesis of males only, but reduce the fertility of both male and female gametes. In contrast, tumour necrosis factor-α does not influence gametogenesis in either sex but impairs zygote

development. Therefore, our experiments demonstrate that immune factors have complex effects on each sex, ranging from reducing the ability of gametocytes to develop into gametes, to affecting the viability of offspring. We incorporate these results into theory to predict how the evolutionary trajectories of parasite sex ratio strategies are shaped by sex differences in gamete production, fertility and offspring development. We show that medical interventions targeting offspring development are more likely to be 'evolution-proof' than interventions directed at killing males or females. Given the drive to develop medical interventions that interfere with parasite mating, our data and theoretical models have important implications.

4.2. INTRODUCTION

Malaria parasites are obliged to undertake a single round of sexual reproduction in the mosquito vector before they can transmit to new hosts, making this stage of their life-cycle a potential target for medical interventions (Paul et al. 2003; Saul 2008). The success of interventions aiming to disrupt mating success will depend upon a variety of epidemiological parameters (e.g. transmission intensity/seasonality), but will also be strongly determined by the parasites' behavioural and evolutionary responses (Paul et al. 2003; Smith et al. 2007; Saul 2008). Current candidates for transmission-blocking vaccines (TBV) involve targeting proteins, expressed on the surface of sexual stages, that are essential for the fertility of males (e.g. P48/45 and P230) (Carter 2001; van Dijk et al. 2001; Outchkourov et al. 2008; Chowdhury et al. 2009; van Dijk et al. 2010). However, theory predicts that the efficacy of a vaccine that reduces the fertility of one sex may be eroded if parasites respond by adjusting their sex ratios in favour of the targeted sex. The study of sex allocation has been one of the most successful areas of evolutionary biology, with empirical data matching clear theoretical predictions across a variety of taxa (West 2010). Before describing evolutionary theory for sex allocation strategies we outline the relevant aspects of *Plasmodium* mating biology.

Every asexual replication cycle, a small proportion of parasites differentiate into male and female sexual stages – termed gametocytes – which are developmentally arrested gamete precursors (Taylor and Read 1997; Talman et al. 2004). Gametogenesis of both sexes begins as soon as gametocytes are taken up in a mosquito blood meal, fertilization occurs within 30 minutes, and zygotes develop into the stages infective to vectors (ookinetes) after 18-20 hours (Alano and Carter 1990; Vaughan 2007). To differentiate into gametes, gametocytes must leave the relative safety of their red blood cells (RBCs), becoming exposed to host- and mosquito-derived factors that can block mating (Alano and Carter 1990). Males are expected to be more vulnerable than females to transmission-blocking factors due to their more complex gametogenesis and mating activities (West et al. 2001; Gardner et al. 2003). Whereas female gametocytes only have to leave their RBCs to become gametes, male gametogenesis also includes three rounds of mitosis and flagellum construction to produce a (rarely achieved) maximum of eight 'sperm-like' gametes (Sinden 1983a; Janse et al. 1986; Sinden 1998; Schall 2000; Reece et al. 2008). Mature male and female gametocytes are easily distinguished by their phenotypes as their reproductive roles result in different cellular contents (Mons 1986; Khan et al. 2005). Mature males are terminally differentiated, only having pre-synthesized proteins and machinery for gamete production (e.g. α-tubulin II, cell cycle proteins, dynein) (Sinden 1983b; Talman et al. 2004; Khan et al. 2005). In contrast, mature female gametocytes are prepared for continued development after fertilization, having high levels of ribosomal proteins, mitochondria (which are absent in mature males) and pools of translationally repressed messenger RNAs (mRNAs; similar to P bodies in metazoan oocytes) (Talman et al. 2004; Khan et al. 2005; Mair et al. 2010). Therefore, male and female gametocytes are primed for gametogenesis and zygote development, respectively (Janse and Waters 2004).

Sex allocation is an important fitness-related trait in *Plasmodium* and could play an important role in the response of malaria parasites to medical interventions that aim to reduce mating success (Paul et al. 2000; Reece et al. 2008; Mitri et al. 2009; Reece et al.

2009). Parasites could respond to transmission-blocking interventions by adjusting their sex allocation strategies via two evolutionary processes. First, if conditions within hosts are unpredictable, invariant, or if variation in within-host conditions is not a good proxy for variation in the mating conditions experienced within vectors, parasites evolve fixed (i.e. canalised) sex allocation strategies that reflect the average environment. Second, if in-host conditions reliably predict in-vector conditions, parasites will evolve to facultatively adjust their sex ratios (proportion of male gametocytes) through phenotypic plasticity. In this scenario, if asexual stage parasites detect an increase in a factor (or correlate of) that reduces mating ability in a sex-specific way, parasites will benefit from adjusting the production of male and female gametocytes in response. Given that once parasites are taken up by a vector, no further gametocyte production can occur and gametogenesis and fertilization are completed within 30 minutes, the mating environment within the blood meal is 'imported' from the host. Therefore, the within-host conditions will be good predictors for mating conditions and so facultative sex ratio adjustment is both predicted and observed (West et al. 2001).

Currently, two complementary evolutionary theories predict how and why parasites should adjust their investment into male and female gametocytes to maximise fertilization success. These theories – Fertility Insurance and Local Mate Competition – predict that parasites adjust sex ratios in response to environmental (e.g. transmission-blocking immunity) and social factors (inbreeding rate), respectively (Hamilton 1967; Read et al. 1992; Read et al. 1995; West et al. 2000; West et al. 2001; Nee et al. 2002; West et al. 2002; Gardner et al. 2003). The ability of parasites to facultatively adjust their sex ratios in response to variation in the inbreeding rate has recently been verified (Reece et al. 2008; Reece et al. 2009). Additionally, data also suggest that sex ratios are altered in response to the development of immunity (Reece et al. 2008). Host-derived immune factors make mating challenging for parasites because they can reduce and even block fertilization (Carter et al. 1979; Naotunne et al. 1991). This phenomenon, called 'transmission-blocking immunity' (TBI), has been extensively observed and

documented across a variety of malaria parasite species (Carter et al. 1979; Mendis et al. 1987; Targett 1988; Naotunne et al. 1991; Naotunne et al. 1993; Drakeley et al. 1998; Long et al. 2008). The mechanisms of TBI are varied and include damaging gametocytes, preventing successful gametogenesis (Naotunne et al. 1991; Naotunne et al. 1993; Cao et al. 1998; Long et al. 2008), decreasing the ability of gametes to interact (Carter et al. 1979; Mendis and Targett 1981) and preventing post-fertilization development (Targett 1988; Luckhart et al. 1998). Fertility Insurance predicts that when hosts mount an immune response, the fertility of male gametocytes and/or gametes is most affected, therefore parasites should produce more males to compensate (West et al. 2001; Gardner et al. 2003).

Two lines of empirical data support this prediction. First, Paul *et al*. (2000) showed that *P. gallinaceum* and *P. vinckei* increase their sex ratio in response to erythropoiesis, which is thought to act as a cue for the appearance of TBI factors. Second, Reece *et al*. (2008) provided indirect support by suggesting that sex ratio variation observed during infections of different *P. chabaudi* genotypes is a mechanism to ensure fertility in face of within-host competition, host anaemia and TBI factors. Fertility Insurance currently provides the best explanation for the observed within-infection variation in the sex ratios of malaria parasites. However, the theory is based upon the untested assumption that TBI factors reduce the fertility of males more than females. Here we provide the first direct test of this key assumption by investigating whether reactive nitrogen species and pro-inflammatory cytokines, influence gametogenesis, gamete fertility and ookinete production.

Levels of reactive nitrogen species (RNS) and pro-inflammatory cytokines vary during malaria infections. These immune factors, which are ubiquitous components of the innate immune system, have been specifically implied in the sudden loss of infectivity to vectors that occurs during paroxysms and infection crisis (Naotunne et al. 1993; Long et al. 2008). Specifically, tumour necrosis factor-α (TNF-α) is a potent pro-inflammatory

cytokine and several studies have revealed a role for this cytokine in mediating the killing of *Plasmodium* gametocytes, across a variety of host-parasite systems (Naotunne et al. 1991; Karunaweera et al. 1992; Long et al. 2008). This could occur through the stimulation of phagocytosis and nitric oxide (NO) production by white blood cells (Naotunne et al. 1993; Tracey and Cerami 1994; Muniz-Junqueira et al. 2001), as these are capable of phagocytosing opsonized gametes in the mosquito midgut (Lensen et al. 1997) and the inhibition of NO synthesis by white blood cells reduces in 60% the inactivation of *P. falciparum* and *P. vivax* gametocytes (Motard et al. 1993; Naotunne et al. 1993). NO is produced by the enzyme inducible nitric oxide synthase in response to infection, in both hosts and vectors, and is extremely toxic at high doses. NO is a highly reactive molecule, thus a significant extent of the damage it causes is indirect, through the production of RNS (such as peroxynitrite, nitrates, nitrites or S-nitrosothiols) that frequently function as the ultimate effectors (Bogdan 2001). Hereafter, unless otherwise stated, we use the term 'RNS' to refer to NO and its reaction products. During *Plasmodium* infections, RNS appears to impair asexual replication, gametogenesis and zygote development (Naotunne et al. 1993; Cao et al. 1998; Luckhart et al. 1998; Wang et al. 2009). Levels of RNS increase during *P. yoelii* infections and reduce ookinete production when either gametocytes or gametes are exposed (Cao et al. 1998). Furthermore, RNS have been shown to induce the programmed cell death of *P. berghei* ookinetes (Ali et al. 2010) and to extensively reduce *P. berghei* oocyst burdens in *Anopheles* mosquitoes (Luckhart et al. 1998). This is, at least in part, the result of a pro-inflammatory response, in which host cytokines induce the mosquito to increase NO (and therefore RNS) production (Luckhart et al. 2003).

Here, we use the rodent malaria parasite *Plasmodium berghei* to conduct a series of experiments to investigate how RNS and TNF-α influence mating success and ookinete production and develop theoretical models that predict the evolution of sex allocation strategies, given the effects observed in our experiments. Therefore, we use these immune manipulations as 'proof-of-principle' for other factors with similar effects on

the sexual reproduction and transmission of malaria parasites. Specifically, we test whether: (1) RNS and TNF-α have dose dependent effects on male gametogenesis (exflagellation) and ookinete production; (2) exposure of male and female gametocytes to both RNS and TNF-α influences their sexual development; (3) the greater effect of RNS we observe on male gametogenesis results in sex-specific fertility effects; and (4) the observed effects of RNS depend on the developmental stage at which parasites are exposed. Our results reveal that RNS reduces male but not female gametogenesis and impairs the fertility of both sexes, whereas TNF-α only affects zygote development. The relative importance of reduced gametogenesis, impaired mating ability and reduced post-mating development have not been explicitly considered by Fertility Insurance theory. Therefore we develop a new mathematical model to derive predictions for how the effects of immune factors generated naturally or by a medical intervention are likely to impact upon parasite sex ratio evolution (a schematic of the biological effects included in the model is presented in Figure 4.1).

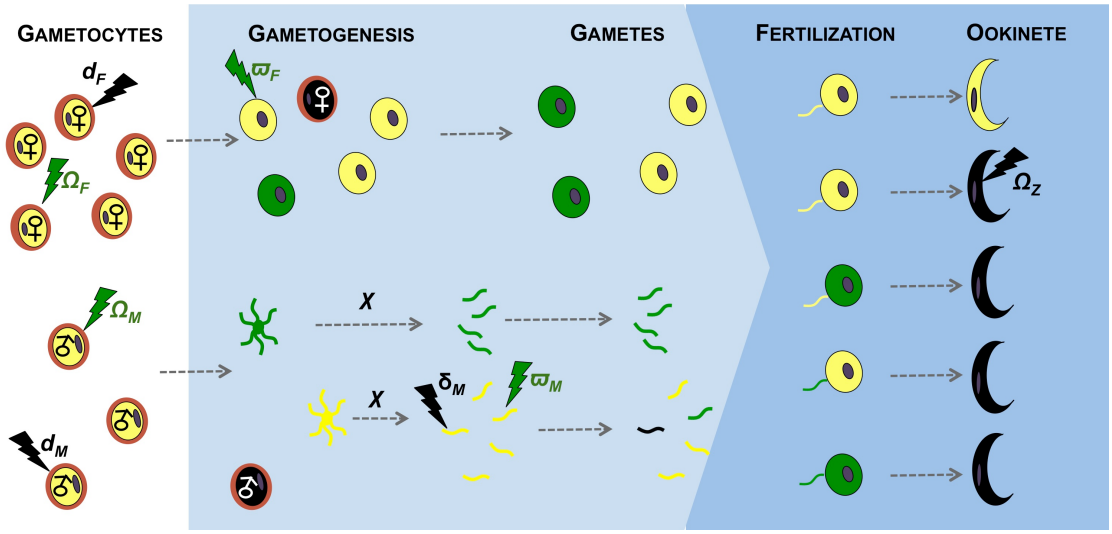

Figure 4.1 Effects of immunity on gametogenesis and fertility of malaria parasites.

The effects of transmission-blocking immune factors on the sexual development of malaria parasites investigated in our model. Female and male gametocytes circulating in the host (white background) undergo gametogenesis when taken up by a mosquito vector (blue background). Each male gametocyte differentiates into χ gametes ($\chi \leq 8$) and each female gametocyte produces one gamete. Male gametes locate and fertilise female gametes, and the resulting zygotes develop into ookinetes. Immune factors circulating in the host can act on males and females throughout their sexual development, from gametocytes to zygotes. The developmental stages of females are shown above the stages of males and each individual gametocyte/gamete is shown in the same relative position throughout development. The effects of immune factors (lighting) on sexual stages can either be cryptic (i.e. render gametocytes/gametes dysfunctional; green), or fatal (i.e. gametocytes/gametes die; black). Healthy, unaffected, parasites are represented in yellow, dysfunctional parasites in green, and dead parasites in black. Immune factors kill female gametocytes with probability d_F and male gametocytes or gametes with probabilities d_M or δ_M, respectively. Dead sexual stages do not participate further in the mating pool.(cont.)

Figure 4.1 (cont.) Immune factors render female gametocytes and gametes dysfunctional with probabilities Ω_F and ϖ_F respectively, and male gametocytes and gametes with probabilities Ω_M and ϖ_M, respectively. Dysfunctional gametocytes/gametes participate in the mating pool and can be fertilized as for healthy gametes, however zygotes are unviable and die before reaching the ookinete stage. Immune factors can also directly lead to zygote death with probability Ω_Z. All possible fertilization scenarios are represented: mating between two healthy gametes, mating between one healthy and one dysfunctional gamete and mating between two dysfunctional gametes.

4.3. METHODS

4.3.1. Hosts and parasites

We maintained MF1 mice, aged 8-10 weeks (Harlan-Olac, UK; or in house supplier, University of Edinburgh), on *ad libitum* food (RM3(P), DBM Scotland Ltd, UK) and water (supplemented with 0.05% PABA to enhance parasite growth), with a 12 hour light cycle, at 21 ºC. We initiated infections by intra-peritoneal inoculation of 10^7 parasitized RBCs in 100 µl carrier consisting of 50% Ringers (27 mM KCl, 27 mM $CaCl_2$, 0.15 M NaCl), 47.5% heat-inactivated foetal bovine serum and 2.5% heparin (5 units ml^{-1}). For experiments 1 and 2, we inoculated female mice, previously (day -3 or -4) treated with 60 mg/kg of phenylhydrazine (PHZ), with *P. berghei* line 820 (Ponzi et al. 2009). For experiment 3 we inoculated male mice (PHZ treatment: 125 mg/Kg, day -2) with one of two *P. berghei* KO lines: *PbΔp48/45* or *PbΔp47* (van Dijk et al. 2001; Khan et al. 2005; van Dijk et al. 2010). We treated mice with PHZ because the resulting release of young RBCs increases gametocyte production in *P. berghei*, which maximises the number of gametocytes that can be harvested for *in vitro* mating experiments (Gautret et al. 1997). For each experiment, parasites were collected from mice on day 3 or 4 post-infection, and each infection contributed parasites to all treatments to control for any potentially confounding influences of differences between infections.

4.3.2. Experimental design

The aim of our experiments was to investigate how RNS and TNF-α affect gametogenesis, fertilization success and zygote development (i.e. ookinete production) of *P. berghei*. In order to do so we developed a series of *in-vitro* experiments in which we manipulated the levels of RNS and TNF-α present in culture at different stages of the sexual development of *P. berghei*.

All the experiments we describe below were performed *in vitro*, using gametocytes harvested form *Plasmodium berghei* infected mice. Parasites were either cultured in conditions that 'mimicked the vector' (in which they immediately became activated and underwent gametogenesis and mating; media at pH 8 and 21 ºC), or conditions that 'mimicked the host' (in which gametocytes remained developmentally arrested; pH 7.25, 37 ºC). Parasites cultured in host mimicking conditions became activated and underwent gametogenesis if subsequently exposed to vector mimicking conditions. The culture media we used follows what is described in (Reece et al. 2008). We exposed parasites to RNS and TNF-α treatments in 1 ml cultures with 15 or 20 µl parasitized blood.

4.3.2.1. Immunity manipulations

We manipulated RNS and TNF-α with the following chemicals: recombinant mouse TNF-α (Sigma, UK), L-ana (L-Arginine p-nitroanilide dihydrochloride; Sigma, UK) and SIN-1 (3-morpholinosydnonimine hydrochloride; Sigma, UK), which were all dissolved in phospate buffered saline. L-ana is a specific inhibitor of the activity of the enzyme inducible nitric oxide synthase, an enzyme which becomes active in response to infection. SIN-1 donates NO and/or superoxide, in solution, at different rates depending on the specific conditions in which SIN-1 is incubated (Feelisch et al. 1989; Noack and Feelisch 1989; Singh et al. 1999). However, given that superoxide and NO react with

each other at an extremely fast rate to produce peroxynitrite ($ONOO^-$), SIN-1 is likely to act as a donor of either NO or peroxynitrite, depending on the rates at which SIN-1 generates NO and superoxide (Singh et al. 1999). The oxygen concentration of the solution is one of the major determinants of whether SIN-1 behaves as a NO or peroxynitrite donor, donating mostly NO in anaerobic conditions and peroxynitrite in aerobic conditions (Singh et al. 1999). In our cultures, oxygen concentrations were in-between fully anaerobic and aerobic conditions, as parasites were incubated in closed 1.5ml tubes. Biological agents, such as human plasma or heme proteins, which are similar to components of our cultures (e.g. mouse plasma, haemoglobin) increase the capacity of SIN-1 to donate NO (Singh et al. 1999). Furthermore, as peroxynitrite can react to produce several RNS (e.g. nitrite, nitrate, S nitrosothiols or nitrosyl-metal complexes) and as we did not measure the specific contributions of each of these factors, we use the term RNS to refer to the factors present in cultures exposed to SIN-1 (Bogdan 2001; Hurd and Carter 2004; Novo and Parola 2008). We did not measure RNS and TNF-α levels in our cultures for three reasons. First, our focus is on testing the effects of RNS and TNF-α on the sexual development of parasites. As our experiments were designed so that each host contributed blood and parasites to all treatment groups in a given experiment, this controls for any variation between infections and ensures that our results are due to the RNS and TNF-α manipulations each culture was subjected to. Second, TNF-α levels were directly manipulated with recombinant mouse TNF-α. Third, we are not aware of any method that would allow us to measure total levels of the different RNS in small volume cultures.

4.3.2.2. Experiment 1

We first tested whether RNS and TNF-α influence sexual reproduction by exposing parasites to different concentrations of these factors and assaying exflagellation and ookinete production. We set up cultures with vector mimicking media for the following SIN-1 concentrations: 0, 0.00001, 0.0001, 0.001, 0.01, 0.1 and 1 mg/ml (Dea-Ayuela et al. 2009), with 6 mice contributing parasites to each treatment. We tested the following

concentrations of recombinant mouse TNF-α: 0, 0.005, 0.01, 0.5 and 1 μg/ml with 4 mice contributing parasites to each treatment. We recorded the densities of exflagellating males after 15-20 minutes and ookinetes after 18-20 hours using a haemocytometer (Reece et al. 2008).

4.3.2.3. Experiment 2

Experiment 1 indicated negative effects of RNS and TNF-α on exflagellation and ookinete production. Therefore in experiment 2, we investigated whether these factors interacted with each other to further impair gametogenesis and parasite mating success and if these effects depended on the developmental stage at which parasites were exposed (i.e. in host conditions as gametocytes or in vector conditions as gametes). For this set of experiments we used a fully cross-factored design, consisting of the following RNS and TNF-α levels: 1 mg/ml SIN-1 (RNS +), 1 mg/ml of L-ana (RNS -), and presence (TNF-α +) or absence (TNF-α -) of 1 μg/ml recombinant mouse TNF-α. Parasites from each of 20 mice were exposed to all four combinations of treatments.

First, we investigated whether RNS and TNF-α affected gametocytes while in the host. To do this, we incubated parasites for 60 min. in host mimicking conditions with RNS and TNF-α. We then replaced treatment media with vector mimicking media (without RNS or TNF-α manipulations) to stimulate gametogenesis. After 15 min. of incubation in vector mimicking media, we made giemsa stained smears from each culture and quantified the development of male and female gametocytes into gametes using the following classifications: (a) mature gametocytes still inside their RBC, (b) gametocytes that had emerged from the RBC and (c) exflagellating male gametes. We used the following criteria to distinguish the developmental stages of gametogenesis: (1) Mature gametocytes: still inside their RBC; females have blue-purple cytoplasm, small, well defined purple nucleus surrounded by a small nucleolus; males have pink-yellow cytoplasm and large disperse pale-pink nucleus. (2) Emerged females: female gamete

condensed into a more circular shape, without a vacuole, cytoplasm staining a more intense blue and a less obvious nucleolus than in a female gametocyte. (3) Emerged male: male gamete with a large circular nucleus in the centre of the cell surrounded by a ring of cytoplasm. (4) Exflagellating male: emerged male gamete progressed to forming up to 8 flagella that protrude from the cell and stain red-purple (Kawamoto et al. 1991; Kawamoto et al. 1992; Reece et al. 2003). We present the results for this experiment as the proportion of a given developmental stage relative to the total number of observed gametocytes/gametes of the same sex.

Second, we investigated the effects of RNS and TNF-α on exflagellation and ookinete production by incubating parasites in culture media mimicking the vector environment. We recorded the densities of exflagellating males and ookinetes as described for experiment 1.

4.3.2.4. Experiment 3

Experiment 2 indicated that only RNS had a significant effect on gametogenesis. In this experiment we then aimed to test for sex-specific differences in fertility (i.e. whether matings with RNS exposed gametocytes/gametes resulted in fewer ookinetes), when parasites were exposed as gametocytes (in host-mimicking media) or during gametogenesis (in vector-mimicking media). We separately exposed each sex to RNS by using two genetically transformed (knock-out; KO) *P. berghei* lines: *PbΔp48/45* and *PbΔp47* (van Dijk et al. 2001; Khan et al. 2005; van Dijk et al. 2010), which produce unviable male and female gametes, respectively.

We infected 38 mice with *PbΔp47* (n=19) or *PbΔp48/45* (n=19). We set up mating cultures following (Reece et al. 2008), by pairing infections according to proximity of their sex ratios, calculated from the densities of *PbΔp48/45* female gametocytes in giemsa stained smears (using criteria described for Experiment 2) and *PbΔp47*

exflagellating males (as for Experiment 1). To avoid pseudo-replication, each infection was only used in 1 pair. For each pair of mice, we made 8 sets of 1 ml cultures, either with (RNS +) or without (RNS -) $1x10^{-5}$ mg/ml SIN-1, mimicking host (60 min. incubation) or vector conditions (15 min. incubation), to which we added 15µl of parasites from one of the infections in each pair (i.e. *PbΔp48/45* or *PbΔp47*). These single sex cultures provided 'exposed' parasites for fertility testing, and corresponded to the following factorial design: 2 conditions (host/vector) x 2 SIN-1 exposures (RNS +/-) x 2 sexes (male/female). After incubation we replaced media in all cultures with 1 ml vector mimicking media (without any SIN-1 manipulation). While 'exposed' parasites were incubating, we collected 60 µl of blood from each infection's pair and added these 'unexposed' parasites to 4 ml cultures in vector mimicking media (without SIN-1). Each 1 ml culture of the 'exposed' parasites was then added to a 4 ml culture containing its 'unexposed' pair and incubated to produce ookinetes (as for Experiment 1). All the cultures were timed so that 'exposed' parasites were added to the cultures containing their 'unexposed' mates at the same developmental stage. For example, a final 5 ml culture could contain 15 µl of blood from a RNS exposed *PbΔp48/45* infection (in which females are the 'exposed' sex) and 60 µl of blood from a *PbΔp47* infection (in which ~4 times more males are provided as 'unexposed' mates). By providing exposed parasites with a surplus of unexposed mates from the opposite sex, we ensured that fertilization of the 'exposed' parasites was not limited by the availability of gametes from the opposite sex. We also set up cultures in vector mimicking media to verify that 'unexposed' parasites from each line are unable to produce ookinetes on their own. We recorded the densities of ookinetes as described for experiment 1.

4.3.3. Statistical analysis

We used linear mixed effects models (R version 2.7.0; R Development Team Core 2011) because, by treating each infection (or pair of infections in Experiment 3) as a 'random' effect, we can account for problems associated with pseudoreplication arising from repeated measurements of each infection. In order to meet the assumptions made by

parametric tests we arcsine square root transformed response variables where necessary. We minimised models following stepwise deletion of the least significant term and using log-likelihood ratio (χ^2) tests to evaluate the change in model deviance until only significant terms remained, and we present F-ratios for fixed effects remaining in minimal models. We then re-ran minimal models using restricted maximum likelihood to estimate the effect sizes reported in the text. Unless otherwise indicated, data and estimated effect sizes are presented as proportions of the focal parasite stage produced in a given treatment, relative to that produced across all treatments for each infection.

4.3.4. Theoretical model

We incorporate our experimental results into Fertility Insurance theory by developing a mathematical model to explore the impact of transmission-blocking factors on the evolution of parasite sex allocation strategies. Specifically, we examine whether sex ratio adjustment could compensate for transmission-blocking factors with the following effects on males or females: (i) preventing male or female gametocytes from undergoing gametogenesis (as each female gametocyte only produces one gamete, killing of these stages is mathematically equivalent); (ii) blocking the mating ability of male gametes; and (iii) causing damage to gametocytes or gametes such that mating can occur but zygotes are not viable. We term the latter phenomenon, of cryptic damage to gametocytes or gametes that results in a dead zygote, as dysfunction. Note that, although we do not observe all of the effects on all stages and all sexes, we incorporate them all in the model (illustrated in Figure 4.1), as they are theoretical possibilities. Also, our model makes no assumptions about whether parasites evolve fixed (i.e. canalised) or facultative (i.e. plastic) sex allocation strategies.

We assume an infinite host population, divided into infected and uninfected individuals, with infected hosts containing a single infection producing haploid gametocytes that circulate in the blood. We assume that q gametocytes are transferred from host to vector

during blood feeding, and that these gametocytes form a single mating group. The expected proportion of males in the mating group is z, i.e. the sex allocation strategy of the parasite strain that contributed the gametocytes. Hence, the actual number of males is a random variable $\alpha \sim Bi(q,z)$ (i.e. binomially distributed with q trials and probability of success z). Consequently, the number of female gametocytes is q-α. Male and female gametocytes are killed with probability d_M and d_F respectively, leaving $\Gamma \sim Bi(\alpha,1\text{-}d_M)$ surviving males and $\phi \sim Bi(q\text{-}\alpha,1\text{-}d_F)$ surviving females. We assume every surviving male produces χ gametes, and every surviving female produces a single gamete. We consider that male gametes are killed with probability δ_M, and hence $\gamma \sim Bi(\chi\Gamma,1\text{-}\delta_M)$ male gametes enter the mating pool. We assume that all ϕ female gametes enter the mating pool (death of female gametes is formally equivalent to that of female gametocytes, and hence is implicitly included in the parameter d_F). Therefore, the number of zygotes is equal to the number of gametes of the limiting sex, i.e. $\zeta = min(\gamma,\phi)$. Finally, we assume that only a proportion p of zygotes are viable, due to either: (a) factors that kill each zygote with probability Ω_Z; (b) factors acting on gametocytes resulting in the production of dysfunctional gametes at rate Ω_M for males and Ω_F for females; or (c) factors acting on gametes and causing them to become dysfunctional at rate ϖ_M for males and ϖ_F for females, i.e. $p = (1\text{-}\Omega_Z)(1\text{-}\Omega_M)(1\text{-}\Omega_F)(1\text{-}\varpi_M)(1\text{-}\varpi_F)$. Inviable zygotes will result when one or both of the parental gametes are dysfunctional. Hence, the number of viable zygotes produced by the mating group is $W = \zeta p$, and this is our measure of fitness (Read et al. 1992; West et al. 2002; Gardner et al. 2003).

4.4. RESULTS

4.4.1. Experiment 1: dose-dependent effects of RNS and TNF-α

We first explored whether RNS and TNF-α affect exflagellation and ookinete production and if this is dose dependent. Our results show that increasing concentrations of SIN-1 caused a significant linear decrease in the densities of exflagellating males ($F_{(1,35)} = 16.28$, $P < 0.0001$; transformed $y = 0.16-0.10x$) and ookinetes ($F_{(1,35)} = 25.86$, $P < 0.0001$; transformed $y = 0.17-0.18x$). Similarly, TNF-α also caused a significant linear decrease in the densities of exflagellating males ($F_{(1,15)} = 6.83$, $P = 0.012$; $y = 0.23-0.09x$) and ookinetes ($F_{(1,15)} = 17.53$, $P < 0.0001$; transformed $y = 0.54-0.37x$).

4.4.2. Experiment 2: effects of RNS and TNF-α on gametogenesis and ookinete production

In this experiment, we aimed to test whether RNS and TNF-α could interact to further reduce gametogenesis and fertilization rates. Moreover, we tested if the effects of these factors depended on the developmental stage at which parasites were exposed (i.e. in host or vector conditions).

First, when we exposed parasites to RNS and/or TNF-α in host mimicking media (Figure 4.2), the proportion of emerged female gametocytes was not significantly influenced by either RNS ($\chi^2_1 = 2.72$, $P = 0.099$) or TNF-α ($\chi^2_1 = 0.12$, $P = 0.731$; or their interaction $\chi^2_1 = 3.38$, $P = 0.066$). In contrast, the proportion of male gametocytes that emerged from RBCs was significantly reduced by RNS ($F_{(1, 59)} = 81.29$; $P < 0.0001$; mean 'RNS -' 0.55 ± 0.02; 'RNS +' 0.32 ± 0.02) but not by TNF-α ($\chi^2_1 = 0.16$, $P = 0.689$; or their interaction $\chi^2_1 < 0.01$, $P = 0.982$). Similarly, the ability of males to exflagellate was significantly reduced by RNS ($F_{(1, 59)} = 33.40$; $P < 0.0001$; mean 'RNS-' 0.15 ± 0.01; 'RNS +' 0.09 ± 0.01) but not by TNF-α ($\chi^2_1 = 0.85$, $P = 0.36$; or their interaction $\chi^2_1 = 0.02$, $P = 0.885$).

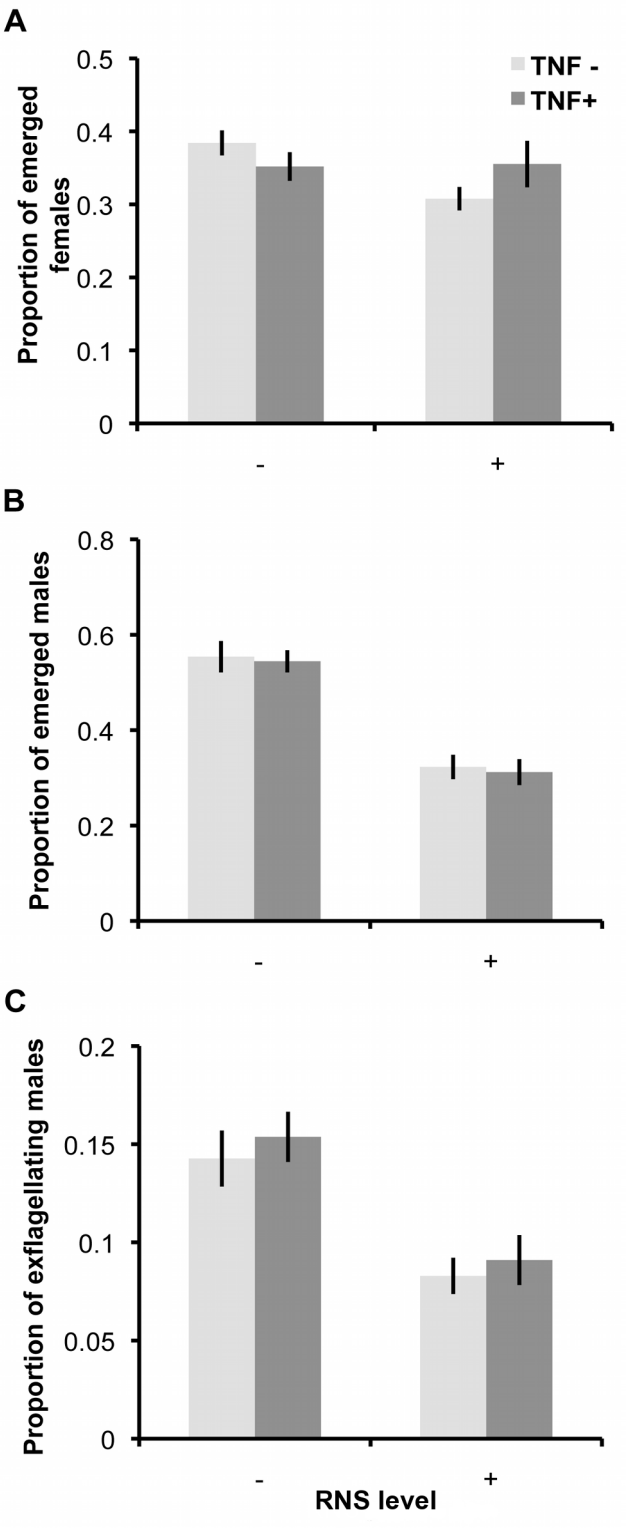
A
TNF -
TNF+
Proportion of emerged females
0.5
0.4
0.3
0.2
0.1
0
-
+
B
Proportion of emerged males
0.8
0.6
0.4
0.2
0
-
+
C
Proportion of exflagellating males
0.2
0.15
0.1
0.05
0
-
+
RNS level

Figure 4.2 Ability of gametocytes to undergo gametogenesis after exposure to RNS and TNF-α (page 112).

Mean (± S.E.) proportion (n = 20) of emerged female gametes (A), emerged male gametocytes (B), and exflagellating male gametes (C), relative to the total number of male or female gametocytes/gametes observed, when gametocytes are exposed to immune factors during incubation in 'host conditions' and then activated in un-manipulated 'vector conditions' media.

Second, in line with the results from our previous experiments, when we incubated parasites in vector mimicking conditions with RNS and/or TNF-α (Figure 4.3), we observed that the proportion of exflagellating males was significantly reduced by RNS ($F_{(1, 45)} = 11.24$, P = 0.002; mean 'RNS -' 0.32 ± 0.06; 'RNS +' 0.12 ± 0.03). This effect was enhanced by TNF-α (interaction: $F_{(1, 45)} = 6.67$, P = 0.014) but in the absence of RNS, TNF-α had no significant effect ($F_{(1, 45)} = 1.90$, P = 0.175). Conversely, the effect of RNS and TNF-α on ookinete production depended on each others presence (interaction $F_{(1, 24)} = 14.91$, P = 0.001). Specifically, ookinete production was reduced by TNF-α but only in the absence of RNS (mean 'TNF-α-' 0.41 ± 0.06; 'TNF-α+' 0.17 ± 0.07), whereas RNS reduced ookinete production but only when TNF-α was absent (mean 'RNS -' 0.41 ± 0.06; 'RNS +' 0.09 ± 0.05).

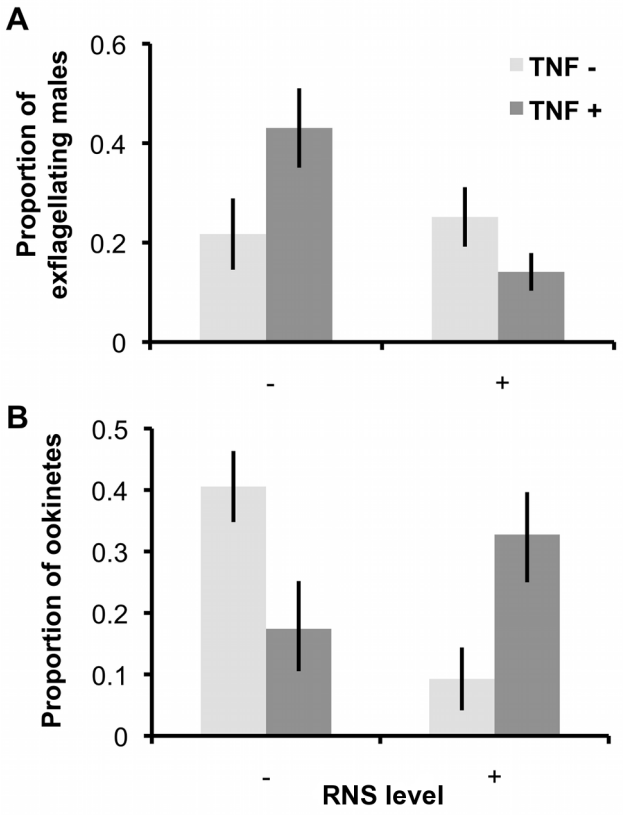

Figure 4.3 Exflagellation rates and ookinete production after exposure to RNS and TNF-α during gametogenesis.

Mean (± S.E.) proportion of exflagellating male gametes (A; n=16) or ookinetes (B; n=9) produced when parasites are exposed to RNS and TNF-α during gametogenesis (in-vector conditions media). Proportions are relative to the total number of exflagellating male gametes or ookinetes produced from each infection, across treatments.

4.4.3. Experiment 3: sex-specific effects of RNS on fertility

In this experiment we aimed to test whether RNS could have sex-specific effects on fertility (i.e. ookinete production) and whether this depended on the stage at which parasites were exposed to RNS (i.e. in host as gametocytes or in vector as gametes;

Figure 4.4). We observed that RNS exposure significantly reduced fertility of both males and females regardless of whether parasites were exposed as gametocytes or during gametogenesis ($F_{(1,131)}$ = 15.87, P = 0.0001; mean 'RNS -' 0.30 ± 0.02; 'RNS +' 0.20 ± 0.02). In contrast to our predictions, RNS did not have sex-specific effects (treatment:sex interaction: χ^2_1 = 0.023, P = 0.88), nor was this effect influenced by exposing parasites to RNS in host- or vector-mimicking environments (treatment:environment interaction: χ^2_1 = 0.366, P = 0.55). However, across all treatments, parasites exposed in host conditions produced significantly more ookinetes than those exposed in vector conditions ($F_{(1,131)}$ = 10.19, P = 0.0018; mean 'Host' 0.29 ± 0.02; 'Vector' 0.21 ± 0.02).

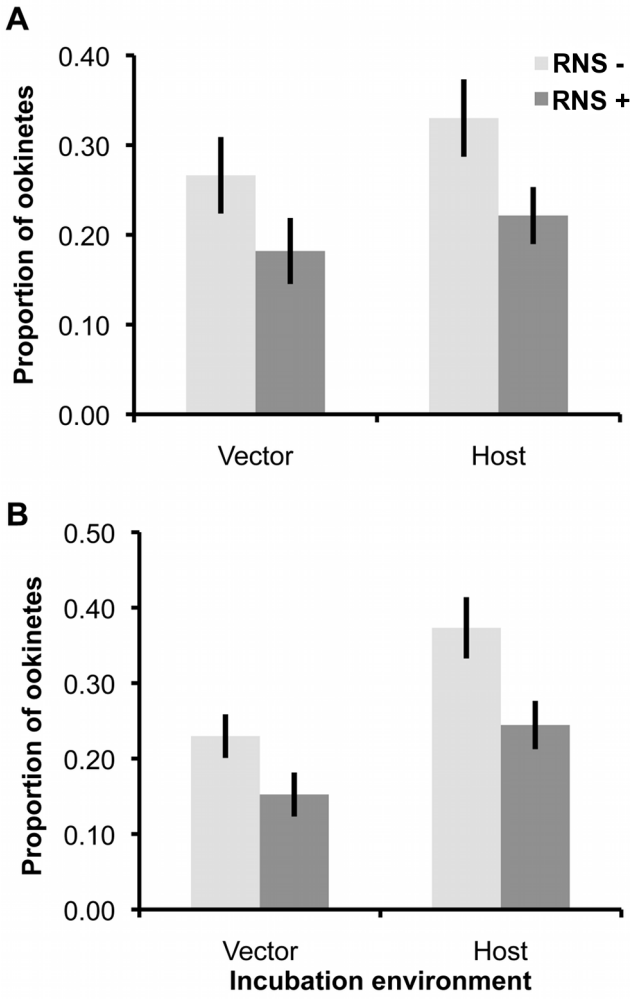

Figure 4.4 Ookinete production after exposure of males or females to RNS, before or during gametogenesis.

Mean (± S.E.) proportion (n=19) of ookinetes produced, when females (A) or males (B) are exposed to RNS as gametocytes (in-host conditions media) or during gametogenesis (in-vector conditions media). Proportions are relative to the total number of ookinetes produced by the focal sex from each pair of infections.

4.4.4. Theoretical model

We incorporate the results above into Fertility Insurance theory as described in the methods and as illustrated in Figure 4.1.

First, we show that all zygote mortality effects (i.e. treatments leading to $0 < p < 1$) have no impact on the evolutionarily stable (ES) sex ratio (Maynard Smith and Price 1973; Maynard Smith 1982). We write $W = \zeta(z)\ p$, i.e. fitness is the product of zygote production and zygote viability, where zygote production depends upon sex ratio but zygote viability does not. The direction of selection is given by the derivative of fitness with respect to sex ratio (Taylor 1996), and this 'marginal fitness' is $dW/dz = (d\zeta/dz)p$. The ES sex ratio z^* satisfies $dW/dz|_{z=z^*} = 0$, i.e. selection does not favour an increase or decrease in sex ratio when the population is at the ES sex ratio, and this is equivalent to the condition $d\zeta/dz|_{z=z^*} = 0$ for all $p>0$. Since ζ is not a function of p, it follows that z^* is not a function of p (and hence is not a function of Ω_Z, Ω_M, Ω_F, ϖ_M or ϖ_F; see Methods and Figure 4.1 for symbol definitions). Therefore, treatments that simply impact upon the viability of zygotes (e.g. cause gametocyte/gamete dysfunction) are not expected to have an evolutionary impact upon parasite sex ratios.

Second, to investigate the impact of model parameters arising from gametocyte or gamete killing on the ES sex ratio, we write an explicit expression for expected fitness:

$$W = \sum_{\alpha=0}^{q}\sum_{\Gamma=0}^{\alpha}\sum_{\gamma=0}^{\chi\Gamma}\sum_{\phi=0}^{q-\alpha}\binom{q}{\alpha} z^{\alpha}(1-z)^{q-\alpha}\binom{\alpha}{\Gamma} {d_M}^{\alpha-\Gamma}(1-d_M)^{\Gamma}\binom{\chi\Gamma}{\gamma} {\delta_M}^{\chi\Gamma-\gamma}(1-\delta_M)^{\gamma}\binom{q-\alpha}{\phi} {d_F}^{q-\alpha-\phi}(1-d_F)^{\phi}\min\{\gamma,\phi\}p \quad (1)$$

The condition $dW/dz|_{z=z^*} = 0$ can be solved numerically for z^* for any numerical parameter set (q, d_M, d_F, δ_M). An exploration of the ES sex ratio z^* across this parameter space is presented in Figures 4.5 and S4.1-3. Specifically, we recover the prediction that the gametocyte ES sex ratio will be biased towards the more limiting sex when factors prevent male or female gametocytes from undergoing gametogenesis or block the mating ability of male gametes.

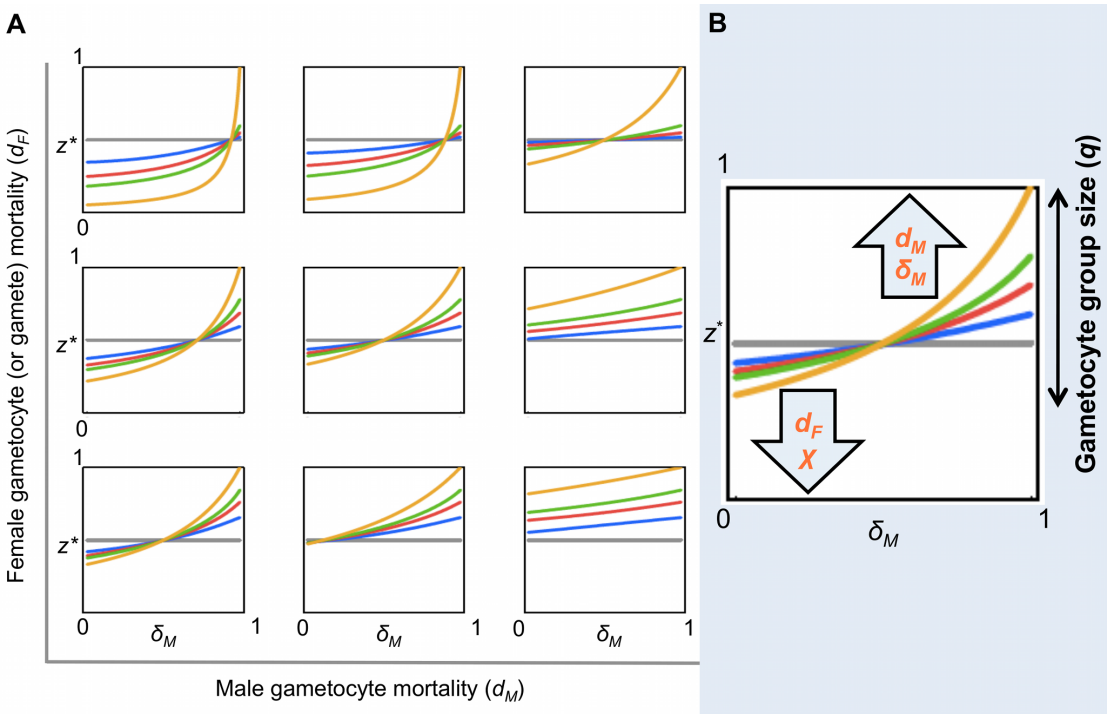

Figure 4.5 Evolutionarily stable sex allocation strategies when sex- and stage-specific mortality rates vary.

Effect of male and female gametocyte mortality and male gamete mortality on the ES gametocyte sex ratio (z^*), for a clonal population, when the number of gametes produced per male gametocyte (χ) is 2 (this fecundity has been estimated for this system by other studies; see Reece et al. 2008). Figures S4.1-3 show similar patterns to Figure 4.5A for χ = 1; 4; 8, respectively. (A) For each plot within the panel, z^* varies with male gamete mortality rate (δ_M). The coloured lines represent different gametocyte group sizes (q): 2 (grey), 5 (blue), 10 (red), 20 (green) and ∞ (yellow). Each plot depicts different parameter combinations of male gametocyte (d_M = 0.1; 0.5; 0.9) and female mortality rate (d_F = 0.1; 0.5; 0.9), with d_M increasing left to right and d_F increasing bottom to top. (B) Cartoon summarizing the effects observed in Figures 4.5A and S4.1-3. The set of possible values for z^* is strongly influenced by q. (cont.)

Figure 4.5 (cont.) The number of gametes of each sex reaching the mating pool (which depends on the mortality parameters and on χ) influences z^* within the constraints determined by q. Within each plot, the effects of δ_M and q on z^* can be clearly observed: the magnitude of sex ratio change increases with q and z^* increases to compensate for higher δ_M. The effects of d_M and d_F can be observed by comparing the points where the lines cross the y axes (i.e. δ_M=0) across the plots: z^* increases along rows with increasing d_M and decreases up the columns with increasing d_F. The effect of χ on z^* can be observed by comparing plots that are in the same position in figures 4.5 and S4.1-4.3: sex ratio becomes more female biased as χ increases.

4.5. Discussion

Evolutionary theory developed to explain the sex allocation strategies of metazoan taxa has enjoyed huge success. Recently, there has been growing interest in whether this theory could be applied to protozoans, particularly malaria parasites (West et al. 2001). The sex ratios of malaria parasites are normally female biased, but extensive variation occurs during the course of infections (2009). Evolutionary theory offers an explanation for this variation and predicts that in-host conditions will influence parasite sex allocation strategies if host-derived immune factors disproportionately reduce the fertility of males relative to females (Hamilton 1967; Read et al. 1992; Read et al. 1995; West et al. 2000; West et al. 2001; Nee et al. 2002; West et al. 2002; Gardner et al. 2003). Here, we tested this assumption by quantifying the effects of two well-known innate TBI factors (RNS and TNF-α) on sexual development and fertility of malaria parasites (West et al. 2002; Gardner et al. 2003). We show that: (1) RNS and TNF-α reduce the densities of exflagellating males and ookinetes in a dose-dependent manner; (2) TNF-α can reduce ookinete densities, but only in the absence of RNS (Figure 4.3); (3) RNS impairs male but not female gametogenesis (Figure 4.2 and 4.3), and reduces the fertility of both males and females independently of whether parasites are exposed as

gametocytes or during gametogenesis (Figure 4.4). We then explored the consequences of our results for parasite sex ratio evolution, by incorporating them into Fertility Insurance theory (Figures 4.1 and 4.5) (West et al. 2002; Gardner et al. 2003). Specifically, our model demonstrates that the ES sex ratio will be biased towards the sex that has a lower number of surviving gametes reaching the mating pool and that the extent of this bias increases as the number of gametocytes in the mating group (q) increases. We also show that factors causing gametes to become dysfunctional (resulting in inviable zygotes) do not affect the ES sex ratio. Below, we discuss the results of our experiments, explain the evolutionary predictions of our model and its implications for the development of transmission-blocking interventions.

4.5.1. RNS, TNF-α and the sexual development of malaria parasites

In our experiments, RNS reduced male but not female gametogenesis while impairing the fertility of both sexes. How can these results be explained? In parasitic infections, high levels of RNS may cause: oxidative damage of DNA (leading to mutations and strand brakes); inhibition of DNA repair and synthesis; inhibition of protein synthesis; inhibition of mitochondrial activity; down- or up-regulation of cytokine (e.g. TNF-α) levels (Clark and Rockett 1996; Bogdan 2001). As described in the introduction, male and female gametocytes are prepared for gametogenesis and zygote development respectively (Janse and Waters 2004). If RNS can impair DNA synthesis and/or microtubule assembly, males would not be able to produce gametes. In contrast, female gametogenesis does not involve these activities and females 'simply' need to leave their RBCs, for which they use the contents of pre-synthesized secretory organelles called osmiophilic bodies (de Koning-Ward et al. 2008). Therefore, whilst female gametogenesis and mating *per se* is unlikely to be influenced by RNS, the development of fertilized females into zygotes and ookinetes is likely to be affected. For example, damage to stored mRNA and inhibition of protein synthesis or mitochondrial activity (e.g. cytochrome oxidases) would impair meiosis (at ~3h after fertilization) and zygote development, but not impair fertilization or gametogenesis (Sinden 1983a; Clark and

Rockett 1996; Bogdan 2001). These effects could explain the observed results, because instead of reducing the ability of females to differentiate into gametes, the effects of RNS would be expressed after fertilization (which we term dysfunction) and lead to female-dependent zygote death, resulting in fewer ookinetes. Here we did not identify the causal RNS and their relative contributions. However, this will be important if transmission-blocking interventions cause or mimic the activities of RNS.

Our experiments show that TNF-α consistently reduces ookinete production and whilst we observed a reduction in exflagellation in some experiments, this effect was inconsistent. Why does TNF-α reduce ookinete production? As TNF-α functions are mainly modulatory and need time to develop, it is possible that gametogenesis and mating occur before the effects of TNF-α manifest. Ookinete development takes about 18-20 hours from fertilization and during this time TNF-α could exert its effects, which could also involve the activation of apoptotic-like death (Janse et al. 1985; Hurd and Carter 2004). Recent experiments provide support for our interpretations, as the deletion of genes coding for proteins essential for the storage and stabilization of translationally repressed mRNAs, in female gametocytes/gametes, do not reduce fertilization success, but substantially reduce the differentiation of zygotes into ookinetes (Mair et al. 2006; Mair et al. 2010). Interestingly, deletion of different genes can affect zygotes throughout development, suggesting that damage to stored mRNA could abort zygote development at multiple stages (e.g. before or after meiosis) (Mair et al. 2010).

4.5.2. Evolution of parasite sex allocation strategies: theoretical predictions

The results of our experiments show that TBI factors can affect the sexual development and fertility of male and female parasites and that the stage at which this occurs is sex-specific. As illustrated in Figure 4.1, we incorporated the observed and potential effects of transmission-blocking factors on males and females, at all stages of development, into

Fertility Insurance theory and generated new predictions for the evolution of parasite sex allocation strategies. Our model predicts that the ES gametocyte sex ratio will be insensitive to variation in gametocyte or gamete dysfunction and zygote mortality. This means that treatments that impact upon the viability of zygotes are not expected to have an evolutionary impact upon parasite sex ratios. In contrast, we predict that the best (ES) sex ratio strategy will vary depending on an interaction between gametocyte group size (q), number of gametes formed per male gametocyte ($0 \leq \chi \leq 8$) and gamete and/or gametocyte mortality. Although, our model makes no assumptions about whether parasites achieve an ES sex ratio through the evolution of facultative or fixed sex allocation strategies, facultative sex allocation is predicted for reasons already outlined in the introduction (see section 4.2).

In the context of clonal infections, the ES sex ratio maximises the expected number of viable zygotes, i.e. maximises the expected number of gametes of the minority sex present in the mating pool (this excludes dead gametocytes/gametes, but includes dysfunctional gametocytes/gametes). For an infinite gametocyte group size (i.e. $q \rightarrow \infty$), that behaves deterministically, the ES sex ratio is one that leads to the same number of male and female gametes being present in the mating pool. This is the sex ratio z^* that satisfies $cz^* = 1\text{-}z^*$, i.e. $z^* = 1/(c+1)$, where c is the number of male gametes, able to mate, produced per male gametocyte (Read et al. 1992; Gardner et al. 2003). Thus, the ES sex ratio is female biased if $c>1$, and male biased if $c<1$ (Figures 4.5 and S4.1-3). However, for finite mating groups ($q<\infty$) – that behave stochastically – the expectation of mating success must be calculated over the whole distribution of possible outcomes. This will tend to reduce the extent to which the sex ratio is biased towards the sex favoured in the deterministic case (West et al. 2002; Gardner et al. 2003). For example, in the extreme of a gametocyte group size of two ($q = 2$; the lowest mating group size for which mating success is possible), the ES sex ratio is always $z^* = 0.5$ (regardless of other parameter values), to maximise the probability of both sexes being present (Figures 4.5 and S4.1-3). Additionally, we reveal that, in a small portion of parameter

space – corresponding to very small gametocyte group sizes, low female mortality, and high male gametocyte mortality and fecundity (χ) – fertility insurance can even lead to a sex ratio bias in the opposite direction (i.e. producing a female biased sex ratio, despite the risk of the absence of males in the mating pool; Figures S4.2-3). This non-intuitive result is due to the way stochastic variation in the number of gametocytes of each sex alters the variance as well as the expected number of gametes of each sex that reach the mating pool. Although the conditions under which this occurs are restrictive, they may be met in natural infections, as many individuals carry gametocytes at extremely low densities (Drakeley et al. 2006). In the context of our experiments and assuming parasites can facultatively adjust sex ratios, our model predicts that if q is high enough to allow for sex ratio adjustment, then RNS should induce parasites to increase the production of male gametocytes.

Our data suggest that RNS reduced female fertility by rendering gametocyte/gametes dysfunctional, so that their fertilisation results in the production of unviable zygotes. The reduction in ookinete production by TNF-α could also be due to male or female dysfunction or, more likely, through increasing zygote mortality. Therefore, we examined the influence of gametocyte and gamete dysfunction and zygote mortality on the evolution of parasite sex allocation strategies. We found that the ES gametocyte sex ratio is independent of these factors (i.e. the occurrence of gametocyte/gamete dysfunction and zygote mortality does not change the relative fitness of different sex ratio strategies). Put simply, this suggests that zygote mortality or gametocyte/gamete dysfunction will not impose selection on parasite sex allocation strategies as parasites cannot compensate for the loss of reproductive success through sex ratio adjustment. More broadly, other immune factors, such as antibodies or complement, could also impair the sexual reproduction of malaria parasites and the effects of such factors should be easily interpreted in light of our theoretical models.

To bring our mathematical modelling in line with our experiments we have focused on the importance of mortality and dysfunction throughout the sexual development of malaria parasites. However two additional factors have an important impact in sex allocation strategies of malaria parasites: (1) the inbreeding rate and (2) the rate at which asexually replicating parasites commit to gametocyte production (conversion rate). The effect of inbreeding on the ES sex ratio is well understood, with theory (Local Mate Competition) enjoying strong empirical support (Read et al. 1992; Read et al. 1995; West et al. 2000; West et al. 2001; Nee et al. 2002; Reece et al. 2008). For clonal mating groups, the ES sex ratio strategy is the one that maximises the overall mating success of the infection as the parasites behave as a single, unified decision maker (West et al. 2001; Reece et al. 2009). In contrast, in mixed infections, conflicts between clones occur, such that the ES sex ratio is the one that maximises each individual clone's inclusive fitness and not the overall mating success of the infection (West et al. 2001; Reece et al. 2009). But for the work we present here, extending our model to allow for a finite number of independent clones per host would not change the qualitative results we present. Fertility Insurance theory predicts that if a low conversion rate results in a small number of gametocytes being taken up by the vector (i.e. small q), parasites should produce a less female biased sex ratio than expected by the inbreeding rate alone. This is due to the stochastic risk of too few males being present in the blood meal to fertilize the females when sex ratios are female biased (Gardner et al. 2003). One intuitive solution for this would be to produce more gametocytes. However, given that gametocyte production comes at a cost to asexual replication, parasites face a trade-off between investment in in-host survival and reproduction (i.e. transmission). Increasing gametocyte conversion is a solution that will not always be available and might be impossible when parasites are 'stressed' (e.g. by in-host competition and low doses of anti-malarial drugs) (Mideo and Day 2008; Reece et al. 2010). Therefore, if transmission-blocking interventions also affect asexual stages and reduce in-host survival, parasites are likely to reduce conversion rates and produce fewer gametocytes.

4.5.3. Implications for transmission blocking interventions

Our model reveals that an intervention with a sex-specific effect on mating ability will elicit an evolutionary response. However, sex ratio adjustment cannot fully rescue zygote production, given that an increase in the number of male gametocytes comes at the cost of decreasing the number of female gametocytes. Nevertheless, in a scenario of widespread transmission-blocking vaccination or treatment with gametocidal drugs with a sex-specific effect, natural selection will "compare" the fitness of parasites that do, and do not, adjust their sex allocation strategies, leading to an increase in the frequencies of the former. Therefore, quantifying the impact of sex ratio adjustment on rescuing fertility and thus, fitness is now required. In contrast, our model also reveals that a transmission-blocking factor resulting in zygote mortality or gametocyte/gamete dysfunction will be 'evolution proof' with respect to parasite sex allocation strategies. Therefore, we suggest that current efforts to prevent fertilization by targeting proteins with sex-specific phenotypes, such as P230, P48/45 (involved in gamete attachment) or Pfg377 (female emergence from the RBC), will be less effective than vaccines targeting zygote development (e.g. P28) (Carter 2001; Saxena et al. 2007; de Koning-Ward et al. 2008). An alternative transmission-blocking approach could cause dysfunctional female gametes by targeting the expression of female-specific translationally repressed mRNAs (Mair et al. 2010). Furthermore, a transmission-blocking intervention combining targets for gamete dysfunction and zygote death would minimize possible redundancy effects, which have been observed in several knock-outs of malaria parasites (e.g. P48/45) (van Dijk et al. 2001).

4.5.4. Conclusions

Given the drive to develop transmission-blocking interventions that disrupt sexual reproduction in malaria parasites, there is an urgent need to evaluate how their short- and long-term success will be influenced by parasite mating strategies. Here, we combined experiments with mathematical modelling to predict how transmission-blocking factors

influence parasite sex allocation strategies. Our model predicts that transmission-blocking interventions causing gametocyte/gamete dysfunction and/or zygote mortality will be 'evolution-proof' from the perspective of imposing selection on parasite sex ratio strategies, i.e. parasites may still evolve other strategies or traits to cope with a transmission-blocking intervention, but these will have to be independent of sex allocation. Put simply, understanding the behavioural strategies that parasites have evolved to cope with naturally occurring transmission-blocking immune factors, will inform predictions for how they will respond to a transmission-blocking factor. More broadly, understanding how, when and why parasites respond to changes in their in-host environment will facilitate the development of interventions that induce parasites to make decisions that are suboptimal for their transmission success, but that are clinically or epidemiologically beneficial. For efficient progress, synergy between research directed at evolutionary and mechanistic explanations for parasite traits and strategies is required.

4.6. APPENDIX

Figure S4.1. Evolutionarily stable sex allocation strategies when sex- and stage-specific mortality rates vary (χ=1).

Figure S4.2. Evolutionarily stable sex allocation strategies when sex- and stage-specific mortality rates vary (χ=4).

Figure S4.3. Evolutionarily stable sex allocation strategies when sex- and stage-specific mortality rates vary (χ=8).

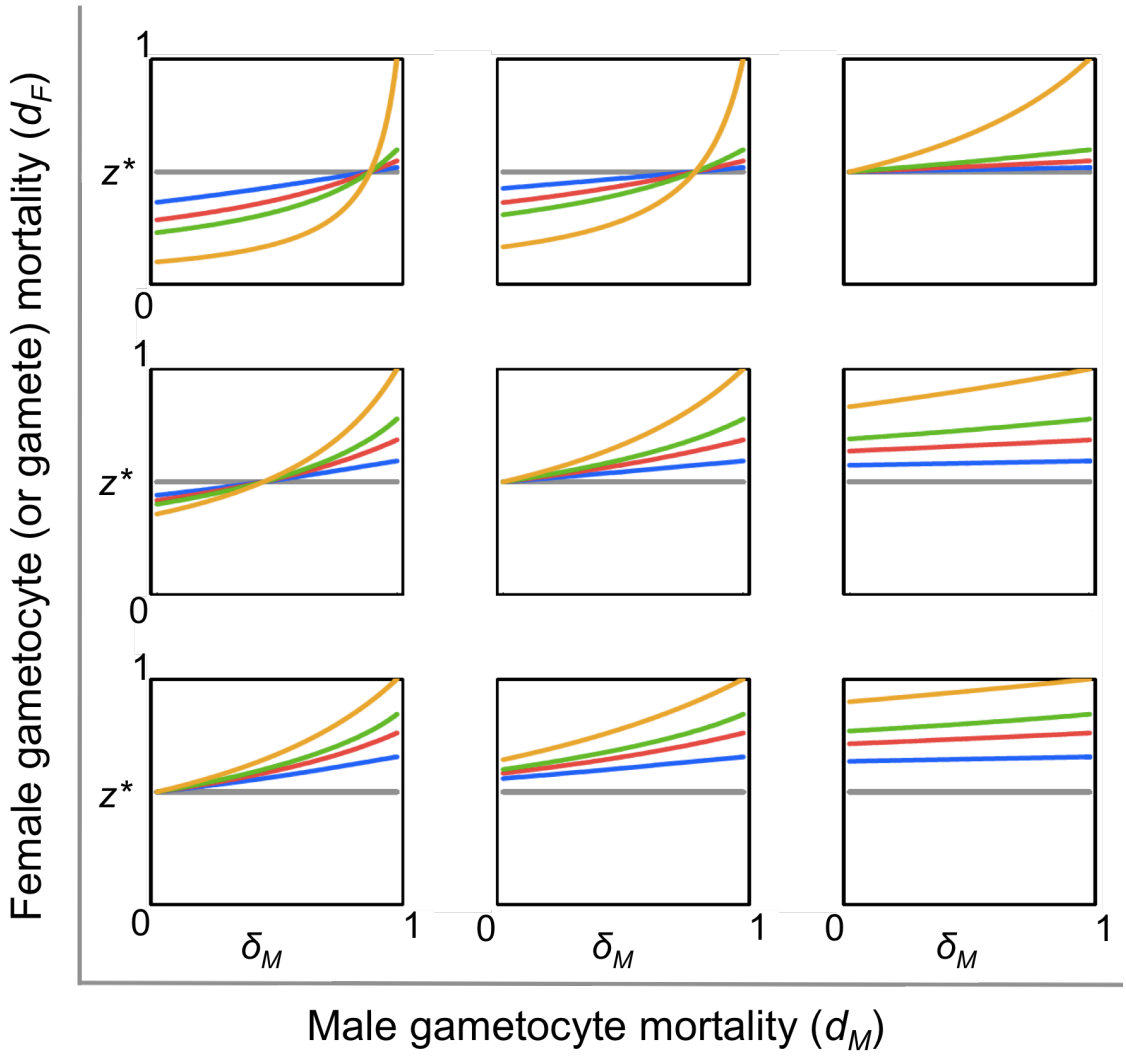

Figure S4.1. Evolutionarily stable sex allocation strategies when sex- and stage-specific mortality rates vary (χ=1). Effect of male and female gametocyte mortality and male gamete mortality on the ES gametocyte sex ratio (z^*), for a clonal population, when the number of gametes per male gametocyte (χ) is 1. On each plot, z^* varies with male gamete mortality rate (δ_M). The coloured lines represent different gametocyte group sizes (q): 2 (grey), 5 (blue), 10 (red), 20 (green) and ∞ (yellow). Every plot depicts different parameter combinations of male gametocyte (d_M = 0.1; 0.5; 0.9) and female mortality rate (d_F = 0.1; 0.5; 0.9), with d_M increasing left to right and d_F increasing bottom to top.

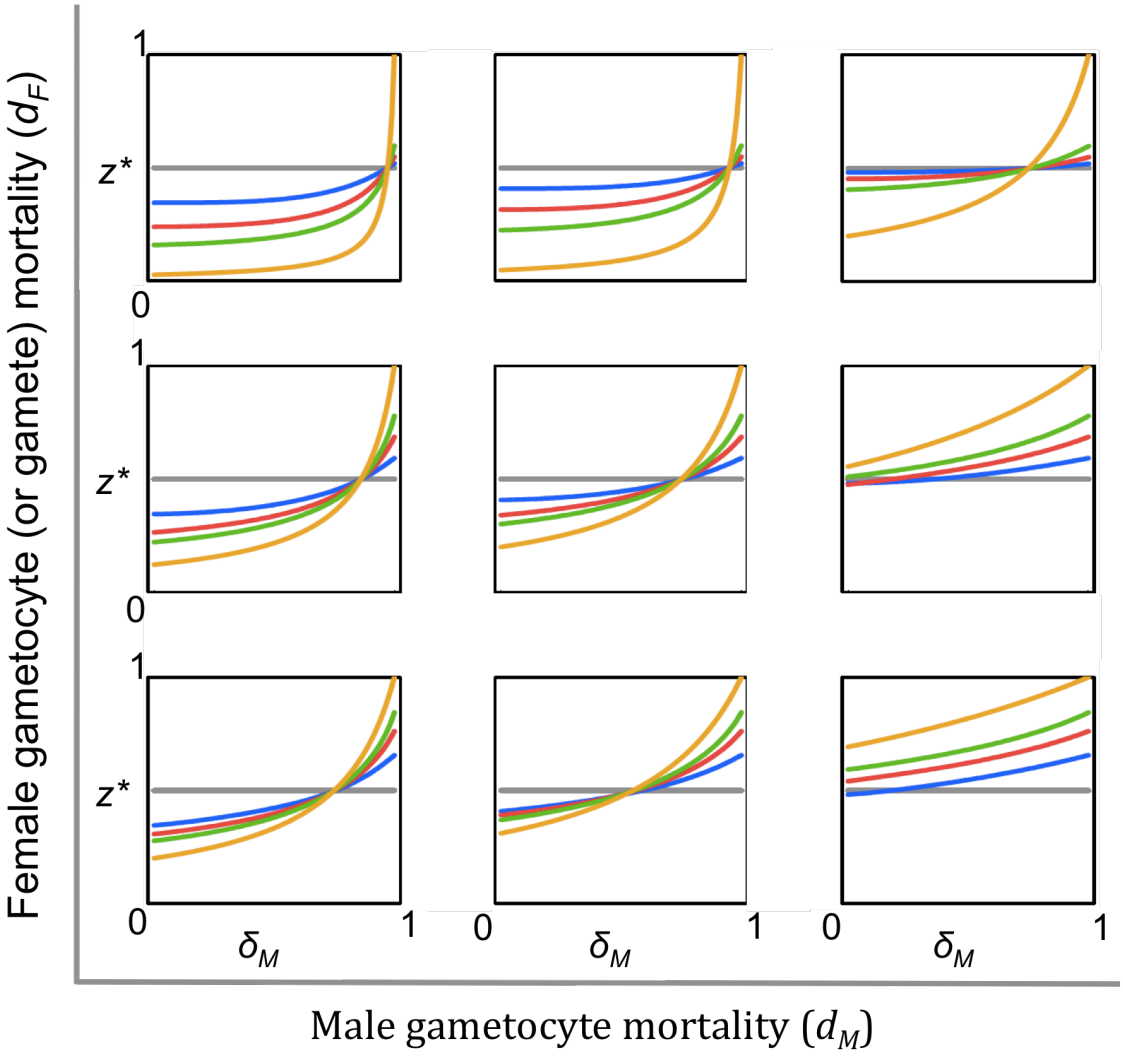

Figure S4.2. Evolutionarily stable sex allocation strategies when sex- and stage-specific mortality rates vary (χ=4). Effect of male and female gametocyte mortality and male gamete mortality on the ES gametocyte sex ratio (z^*), for a clonal population, when the number of gametes per male gametocyte (χ) is 4. On each plot, z^* varies with male gamete mortality rate (δ_M). The coloured lines represent different gametocyte group sizes (q): 2 (grey), 5 (blue), 10 (red), 20 (green) and ∞ (yellow). Every plot depicts different parameter combinations of male gametocyte (d_M = 0.1; 0.5; 0.9) and female mortality rate (d_F = 0.1; 0.5; 0.9), with d_M increasing left to right and d_F increasing bottom to top.

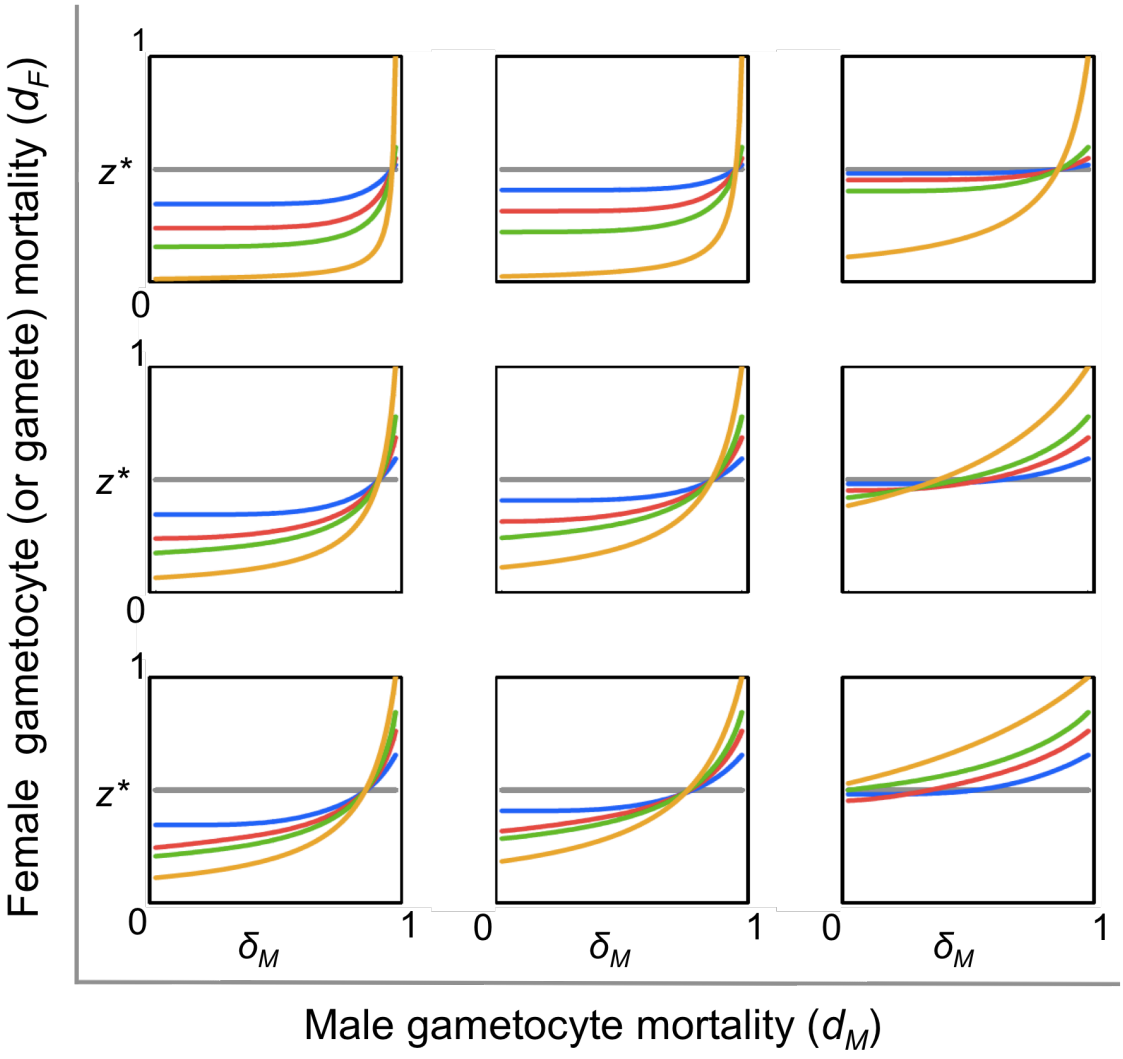

Figure S4.3. Evolutionarily stable sex allocation strategies when sex- and stage-specific mortality rates vary (χ=8). Effect of male and female gametocyte mortality and male gamete mortality on the ES gametocyte sex ratio (z^*), for a clonal population, when the number of gametes per male gametocyte (χ) is 8. On each plot, z^* varies with male gamete mortality rate (δ_M). The coloured lines represent different gametocyte group sizes (q): 2 (grey), 5 (blue), 10 (red), 20 (green) and ∞ (yellow). Every plot depicts different parameter combinations of male gametocyte (d_M = 0.1; 0.5; 0.9) and female mortality rate (d_F = 0.1; 0.5; 0.9), with d_M increasing left to right and d_F increasing bottom to top.

5. Facilitation, apparent competition and virulence in mixed-species infections

5.1. SUMMARY

Distinguishing the contributions that host resources and immunity make to the dynamics of infections is a key question in disease ecology. This is particularly important in the context of mixed infections because theory generally predicts that more virulent parasites are selected for. Virulence may be favoured because faster replication is advantageous when parasites compete for a shared pool of resources and/or when safety in numbers helps parasites survive host immune responses. In malaria infections, competition for shared resources generally occurs in mixed infections containing conspecifics, but this may not be the case for mixed-species infections. This is because different species of malaria parasites differ markedly in the age of red blood cells they are able to infect, with some species being specialists on young (*Plasmodium vivax* or *P. yoelii*) or mature red blood cells (*P. malariae*), while others are generalists (*P. falciparum* or *P. chabaudi*). Even though species with such divergent resource use strategies are often found co-infecting the same host, very little is known about the interactions that occur in mixed-species infections and what are the fitness consequences for both parasites and hosts. Here we use two rodent malaria parasites, *P. chabaudi* and *P. yoelii*, which have similar red blood cell age preferences to important human malarias (*P. falciparum* and *P. vivax)*. We manipulate the within-host environment to dissect how the performance of *P. yoelii* is affected by the presence of *P. chabaudi* and whether resource availability or immunity shape interactions between species. We show that *P. chabaudi* can boost *P. yoelii* above its single species level (i.e. facilitation) and that this is mediated by resource availability. Conversely, *P. yoelii*'s performance can be hindered in mice that were previously cured from a *P. chabaudi* infection (heterologous immunity; apparent competition), but this effect is minor compared to the effect of a previous *P. yoelii* infection (homologous immunity). The development of homologous immunity to *P. yoelii* is density dependent, leading to a trade-off between the performance of

current and future infections. Finally, we show that host mortality is exacerbated in mixed-species infections and suggest this may be due to an inability of the host to achieve the right balance between the production and the destruction of red blood cells when challenged by parasites with different red blood cell age preferences.

5.2. INTRODUCTION

Mixed-species infections are common and these interactions have been shown to promote or inhibit the co-occurrence of other parasite species in the same host (Howard et al. 2001; Graham 2008; Telfer et al. 2010). Such interactions can affect disease severity, parasite fitness and ultimately the prevalence and distribution of different parasite species (McKenzie and Bossert 1997; Piroth et al. 1998; McKenzie and Bossert 1999; Sulkowski et al. 2000; Mayxay et al. 2004; Hartgers and Yazdanbakhsh 2006; Genton et al. 2008; Skinner-Adams et al. 2008; Tjitra et al. 2008). Whilst most research on parasite communities has focused on classifying whether interactions have positive or negative outcomes for the species involved, considerably less attention has been paid to the within-host mechanisms that underpin interactions and ultimately determine their outcomes (Pedersen and Fenton 2007). For example, when interactions are negative, is this due to exploitation competition for the same resource, or by stimulating stronger immune responses and causing apparent competition, or are the interactions direct (via interference competition)? Similarly, positive interactions (e.g. facilitation) could occur if the presence of parasite species A leads to an increase in resource availability for species B, inhibits immune responses that target B, or if products from the metabolism of A benefit the growth of B. Understanding the within-host mechanisms mediating interactions between parasites is essential for predicting parasite evolution (Gilchrist and Sasaki 2002; Pedersen and Fenton 2007; Mideo 2009) and requires experimental systems where the ecology of individual species is well understood, the performance of each co-infecting species can be tracked, and for which resources and immunity can be perturbed. Rodent malaria (*Plasmodium*) species are ideal model systems for this purpose. Here, we use rodent malarias to investigate the roles of resource availability and immunity in determining the outcome of mixed-species interactions.

Disentangling the roles of "top-down" (immunity) and "bottom-up" (resources) processes is essential for understanding the outcome of mixed-species infections and is a key ambition in disease ecology (Haydon et al. 2003; Pedersen and Fenton 2007; Metcalf et al. 2011). In *Plasmodium* infections, immunity to one genotype is able to suppress heterologous genotypes but not as strongly as homologous genotypes (Cheesman et al. 2006), and cross-species immunity has also been observed (Bruce et al. 2000). This suggests a potential role for top-down regulation of mixed-species infections via apparent competition. On the other hand, bottom-up processes could also regulate parasite densities during mixed infections, particularly because there is considerable variation for RBC age preference across species (Reece et al. 2009), which will significantly constrain resource availability. For example, *P. berghei* and *P. yoelii* are restricted to invading immature RBCs (reticulocytes), *P. vinckei* is restricted to mature RBCs (normocytes), and *P. chabaudi* is a generalist perhaps with a slight preference for normocytes (Veins et al. 1971; Killick-Kendrick and Peters 1978; Cromer et al. 2006; Mideo et al. 2008). The RBC age preferences of the rodent malarias are mirrored in the human malaria parasites, with *P. vivax* and *P. ovale* being reticulocyte restricted, *P. malariae* preferring normocytes and *P. falciparum* being more generalist (McKenzie et al. 2001, 2002).

The role of RBC preference in shaping infection dynamics (of single or mixed infections) has received little attention relative to the roles of immune factors (but see McQueen and McKenzie 2004; Reece et al. 2005; Cromer et al. 2006; Mideo et al. 2011). However, RBC preference is important because as infections progress, anaemia develops and the age structure (as well as overall density) of RBCs changes considerably (McQueen and McKenzie 2004). The proportion of RBCs that are reticulocytes in healthy mammalian hosts is usually ~1% but this increases rapidly in response to anaemia caused by parasite exploitation of RBCs (Yap and Stevenson 1994; Savill et al. 2009). Therefore, whilst co-infections between con- or heterospecifics with similar RBC age preference can result in exploitation competition, for heterospecifics with different RBC age preference, facilitation could occur. For example, theory predicts that *P. vivax,* can be facilitated by co-infection with *P. falciparum* because *P. falciparum* enhances anaemia and RBC production,

leading to an increase in reticulocyte availability, which is the preferred resource for *P. vivax* (McQueen and McKenzie 2006). Here, we test this theory using rodent malaria parasites with similar RBC preferences (like *P. vivax, P. yoelii* is reticulocyte restricted and *P. chabaudi* has similar preference to *P. falciparum*). We conduct a series of experiments in which we manipulate the within-host environment in terms of resource availability, heterologous immunity, and the presence/absence of mixed infections. Our experiments follow *P. yoelii* as a focal species to test whether: (a) facilitation can occur in mixed-species infections; (b) facilitation is driven by changes in RBC age structure; (c) if apparent competition plays a role in the dynamics of mixed-infections; (d) facilitation increases the virulence of infections (as also suggested by McQueen and McKenzie 2006); and (e) enhanced performance of *P. yoelii* in primary infections has consequences for its performance in secondary infections. Our results suggest that both facilitation and apparent competition occur, but the frequency of available resources is the main factor determining *P. yoelii* performance. In agreement with recent field studies of *P. vivax-P. falciparum* mixed infections (Genton et al. 2008; Tjitra et al. 2008), we find that mixed infections of naïve hosts are associated with extremely high mortality. Finally, we show that *P. yoelii*'s performance in a secondary challenge depends on its performance during the primary infection.

5.3. MATERIAL AND METHODS

5.3.1. Hosts and parasites

We maintained MF1 male mice, aged 8-10 weeks (Harlan-Olac, UK), on *ad libitum* food (RM3(P), DBM Scotland Ltd, UK) and water (supplemented with 0.05% PABA to enhance parasite growth), with a 12 hour light cycle, at 21 ºC. Mice were randomly allocated to cages with 2-4 animals. When illness reduced the ability to freely move and feed, mice were moved to individual cages with mashed food. We used the two following non-lethal parasite genotypes: *Plasmodium chabaudi chabaudi* AS (AS12476) and *P. yoelii yoelii* 17X Mill Hill (35GA), which were obtained from the WHO Registry of Standard Malaria Parasites in the European

Malaria Reagent Repository, University of Edinburgh. For simplicity, hereafter we refer to these parasite genotypes as *P. chabaudi* and *P. yoelii*.

5.3.2. Experimental designs

5.3.2.1. Experiment 1

The aim of this experiment was to investigate the impact of facilitation (via resource availability) and apparent competition (via heterologous immunity) on the within-host performance of *P. yoelii*. In order to do so, we manipulated the within-host environment to increase levels of immunity, resources, or provide a competitor (*P. chabaudi*) before infecting mice with *P. yoelii* and monitored infections for 26 days. The preparation for each treatment group is illustrated in Figure 5.1 and consisted of:

Single infection (S): we infected mice with a single dose of *P. yoelii*. This treatment serves as the main baseline for *P. yoelii*'s performance.

Mixed-infection (M): we infected mice with a mixed-species infection of *P. yoelii* and *P. chabaudi*. This treatment serves baseline for *P. yoelii*'s performance in a mixed infection.

Apparent-competition (single, AC_S; and mixed, AC_M): we first infected mice with *P. chabaudi*. After 8 days, we cured mice by injecting them with 12mg/kg pyrimethamine (in 50µl DMSO; Sigma, UK) for two consecutive days and then providing pyrimethamine-treated water (7mg/ml) for further 2 days. We allowed mice to recover for 18 days. After this period, we gave mice either a single-species (*P. yoelii; AC_S*) or a mixed-species (*AC_M*) infection. Two days before infecting mice with *P. yoelii*, we confirmed (by qPCR) the absence of *P. chabaudi* parasites in all mice (data not shown). The aim of these treatments was to increase heterologous immunity by the time of *P. yoelii* infection.

Artificial Anaemia (AA): we gave mice an IP injection of 60mg/kg phenylhydrazine (PHZ; dissolved in PBS; Sigma, UK) three days before infecting them with *P. yoelii*. PHZ greatly accelerates the aging of RBCs and their clearance from circulation, which leads to a large and immediate increase in the availability of reticulocytes (the

preferred resource of *P. yoelii*) (Savill et al. 2009). The aim of this treatment was to elevate resource availability by the time of *P. yoelii* infection.

Parasite-Induced Anaemia (PIA): we infected mice with *P. chabaudi* 10 days before they received *P. yoelii*. We chose this time point because *P. chabaudi* AS infections peak between day 8-12 of infection, after which reticulocyte density greatly increases (Yap and Stevenson 1992). The aim of this treatment was to elevate resource availability whilst providing a competitor by the time of *P. yoelii* infection.

We initiated single-species infections by intra-peritoneal (IP) injection of 10^5 parasitized RBCs in 100 μl carrier (following Bell et al. 2006) and mixed-species infections with $2x10^5$ infected RBCs in 200 μl carrier, consisting of 10^5 infected RBCs of each parasite species. All mice in all treatment groups entered the experiment at the same time and all experimental infections were initiated on the same day. To control for any possible effects of the drug treatments, *P. chabaudi* injections or PHZ manipulations, we injected mice in the groups not receiving these treatments with the carrier solution plus uninfected RBCs (to control for parasite injection) or with PBS (to control for PHZ), and we injected all mice with the same dose of pyrimethamine and they all received the same pyrimethamine-treated water.

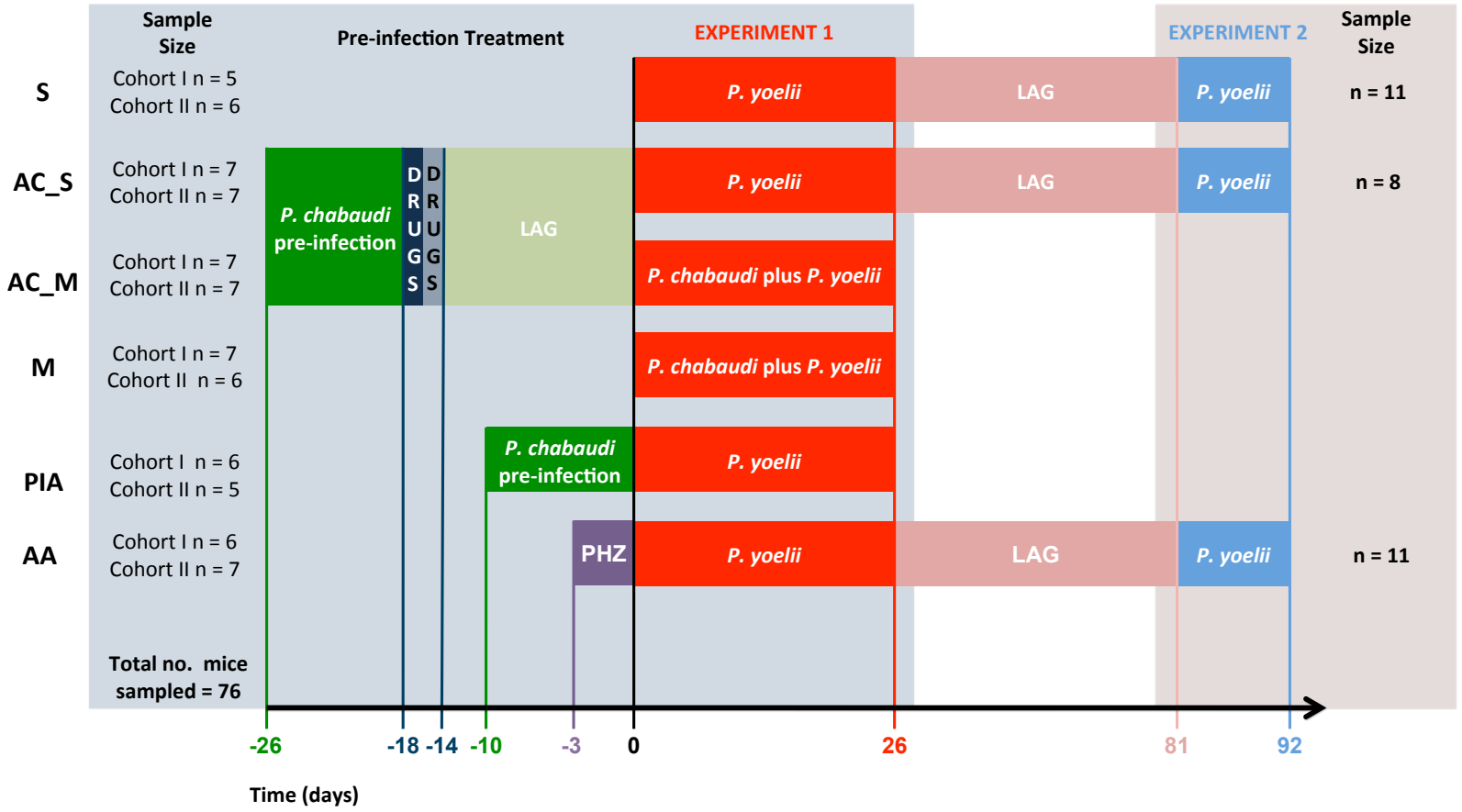

Figure 5.1. Diagram of Experimental Design.

This figure represents a time line of the different procedures carried for experiments 1 and 2. For the full details for each treatment please see section 5.3.2. (cont.)

Figure 5.1 (cont.) All mice began the study together (on day -26) and the different rectangular boxes represent the time windows in which mice were: a) infected with *P. chabaudi* (dark green); b) given a pyramethamine injection (DRUGS; dark blue); c) given pyramethamine diluted in their drinking water (DRUGS; pale blue); d) given a PHZ injection (a single injection on Day -3; purple); e) recovering from infection (LAG; light green and light pink). The experimental infections are either in red (experiment 1) or blue (experiment 2) boxes. Sample sizes, per experiment, treatment and cohort are shown.

5.3.2.2. Experiment 2

In this experiment we tested whether there was a trade-off between the performance of *P. yoelii* in a primary and a secondary infection (i.e. whether parasite density achieved in a primary infection negatively affects parasite density on a secondary infection). To do this, we re-infected a subset of mice from experiment 1 with *P. yoelii*, 55 days after the end of this experiment (infections were initiated as for experiment 1). These mice belonged to the following treatments: S, AC_S and AA. We focussed on these treatment groups to avoid possible confounding effects arising from a mixed *P.yoelii-P. chabaudi* infection. We also infected 5 additional age- and sex-matched naïve mice with *P. yoelii* to create a new S group and verify that parasite performance were not significantly different to that of treatment S in experiment 1 ($F_{(1,14)} = 2.41$, $p = 0.14$). Figure 5.1 illustrates how experiment 2 follows from experiment 1 and the number of mice use in each treatment group.

5.3.3. Data collection

5.3.3.1. Infection sampling

For experiment 1, we sampled mice between day 0 and 26 post-infection (PI). To maximize the number of mice that could be sampled during experiment 1, we randomly assigned mice to two cohorts (I and II) that were sampled on alternative days. For experiment 2, we sampled all mice every day between day 0 and 10 PI. We took samples of tail blood to quantify RBCs by flow cytometry (2µl blood; Beckman

Coulter), reticulocytes by microscopy (thin blood smears) and parasites both by microscopy and qPCR (5µl; only for experiment 1). To quantify proxies for heterologous and homolgous immunity we assayed IgG2a antibody levels (10µl Blood), on day 0 and 15 PI for experiment 1 and on day 0 and 5 PI for experiment 2. The methods used to assay these variables are detailed below. To quantify morbidity, mice in both experiments were weighed daily. We stopped sampling in each experiment when parasitaemia was no longer patent by microscopy for the majority of mice.

5.3.3.2. Microscopy

We stained thin blood smears with 10% Giemsa buffered in 90% phosphate solution for 15min. We used microscopy to determine the number of infected cells and the proportion and density of reticulocytes. Giemsa staining allows us to distinguish between reticulocytes and mature cells (normocytes), as cells stain purple and grey, respectively. It is also possible to distinguish between most stages of *P. yoelii* and *P. chabaudi* using Giemsa stained smears. At the time of sampling, *P. chabaudi* (a synchronous parasite) was present as ring stages but *P. yoelii* is asynchronous so all stages were present.

5.3.3.3. Quantitative PCR

DNA was extracted as described in (Bell et al. 2006), and species-specific PCR assays used to distinguish between *P. chabaudi* and *P. yoelii*. For both species the assay used targets a conserved locus, previously named as common gametocyte gene 1 (PCHAS_062090 for *P. chabaudi* and PY02842 for *P. yoelii* in http://plasmodb.org/) (Khan et al. 2005; Drew and Reece 2007). The assay for *P. chabaudi* has been previously published in (Wargo et al. 2007a) and uses the following design: forward primer 5'-CAC AAT ATA GTA TAA AAG TAG GAC TTG AAA ATA ATA GTA G-3', reverse 5'-GGA ATA TGG GAT ATT GTC AAA GGA TAT AC-3', probe (TaqMan MGB) 5'-6FAM–TTT TCC ACT TAC AAC TCC A-3'. The *P. yoelii* assay was custom made for this experiment by

PrimerDesign, Co and has the following design: forward 5'-CTG TAA GGC CAT TTA AGG GGG-3', reverse 5'- TCA TAG TAC CAT TCT TTT TGC TTT TTC-3', probe (TAMRA) 5'-6FAM-CTA AGT CAT CAT TTA TAA CCT TTA CAG CAT CAT CAT-3'. qPCR reactions were performed as described in (Wargo et al. 2007a) for *P. chabaudi* and following PrimerDesign's guidelines for *P. yoelii*. Briefly, for *P. yoelii*, reactions were set up at a final volume of 20µl, using 5µl DNA, 10µl of 2x TaqMan universal PCR master mix (Applied Biosystems), 4µl of water and 1µl of the primer/probe mix provided by PrimerDesign (cycling conditions were: 95ºC for 10min. and then 50 cycles of 95ºC for 15s and 60ºC for 1min.). We validated both assays before analysing the experimental samples to confirm that: (1) both assays are species-specific, with no sign of cross-reactivity; (2) the sensitivity of each assay is not affected by the density of heterospecifics; (3) both assays show low interassay variation (*P. yoelii*: 0.4-2.8%; *P. chabaudi*: 0.5-0.8%) (Schneider et al. 2005); and (4) the detection limit is low and similar (for both species) at 2-5 and 3-5 parasites/µl for *P. yoelii* and for *P. chabaudi*, respectively.

5.3.3.4. Immunological assays

We measured species-specific antibodies (IgG2a) using enzyme linked immunosorbent assays (ELISA). Antibodies of the IgG2a class have been shown to induce strong protection against malaria in mice (Cavinato et al. 2001). The antigens used were MSP1 for *P. chabaudi* (kindly provided by Karen Fairlie-Clarke) and crude antigen homogenate from parasitized erythrocytes for *P. yoelii*. We used MSP1 for *P. chabaudi* because antibodies against this protein have been shown to correlate with protection in the rodent malaria system (O'Donnell et al. 2001; Burns et al. 2004). We prepared crude antigen homogenate from *P. yoelii* parasitized erythrocytes, following an adapted protocol from (Nie et al. 2009). Briefly, we bled *P. yoelii* infected mice by cardiac puncture into heparinised syringes and diluted blood 1:2 in RPMI medium. This was underlayed in 67% Percoll:RPMI and centrifuged at 1500xg (10min.). We then harvested late stage parasites from the interface, washed them in RPMI and centrifuged at 2000rpm (5 min.). We then incubated parasites in pre-warmed trypsin at 37ºC (to cleave antibodies) and washed

cells twice with RPMI by centrifuging at 2000rpm (5min.) We then re-suspended cells in pre-warmed 0.05% saponin to lyse RBC membranes, centrifuged this at 2000rpm (10min.), washed the supernatant in PBS until this became clear and lysed parasites with 5 cycles of freeze-thawing. Finally we measured protein concentration using a Bradford assay (BioRad).

To measure IgG2a specific against *P. yoelii*, we first coated plates (Immunosorb, NUNC) with 50µl (per well) of 1µg/ml of crude antigen homogenate in carbonate buffer and incubated them overnight at 4ºC. We blocked non-specific binding with 100µl of 5% Marvel:carbonate buffer and incubated this at 37ºC for 2h. We washed wells 5 times with Tris buffered saline with 0.1% Tween (TBST) after each incubation. We then added serum samples at dilutions from 1:100 to 1:800 and incubated plates for 2h at 37ºC. After this, we filled wells with 100µl of 1µg/ml Biotin Rat Anti-Mouse IgG2a detection antibody (BD Biosciences) diluted in TBST:1%Bovine serum albumine (BSA) and incubated plates for 1h at 37ºC. Finally, we diluted extravidin peroxidase 1/8000 in TBST:1%BSA (100µl per well), incubated plates for 30min. at 37ºC and developed samples with 100µl per well of TMB for 20min. We stopped reactions with 100µl of 1M HCl and read plates at 450nm. For the *P. chabaudi* assay we followed the same protocol, with some exceptions: plates were coated with *P. chabaudi* MSP1, non-specific binding was blocked by 5% BSA:carbonate buffer, horseradish peroxidase conjugated goat anti-mouse IgG2a (Southern Biotech) was used as the detection antibody and no extravidin peroxidase step was performed.

5.3.4. Data analysis

5.3.4.1. Treatment effects

We first tested whether our manipulations of the in-host environment had the desired effects, and that mice in cohorts I and II did not differ significantly. To do this we used data collected on day 0 of experiment 1 (Figure 5.2) and fitted linear models with treatment and cohort as explanatory variables for the following response variables: RBC density, proportion of reticulocytes, reticulocyte density, *P. chabaudi*

and *P. yoelii* antibody levels. For variables where treatment was significant, we then used *post-hoc* all pairs Tukey tests to infer which treatments were significantly different from each other.

5.3.4.2. Parasite density data

Whilst qPCR is now the most commonly used method for quantifying *P. chabaudi*, *P. yoelii* infections pose two challenges to the interpretation of parasite counts obtained by this technique that are not problematic for *P. chabaudi* infections. First, *P. yoelii* infections are asynchronous, with RBCs often being infected by schizonts – the later stage of asexual replication in the blood – each containing between 12 and 18 progeny merozoites, i.e. between 12-18 parasite genomes (Killick-Kendrick and Peters 1978). Second, in *P. yoelii* infections, RBCs are often multiply infected. Both of these features of *P. yoelii* mean that the number of parasite genomes in a *P. yoelii* infection does not directly correspond to the number of individual parasites or to the number of infected cells. This means that qPCR counts will often overestimate the number of parasites as well as the amount of resources (i.e. RBCs) being used by them. Microscopy – in which individually infected RBC are identified and quantified – can overcome these problems. However, whilst it is possible to differentiate between some stages of *P. yoelii* and *P. chabaudi* parasites in a mixed infection, this is difficult when examining ring stages and so independent counts of each species in mixed infections may not be as accurate as for single species infections.

Given these issues we decided to use qPCR to obtain data for both cohorts and use microscopy to obtain data from cohort I (between days 3 and 23) and compare the results of the two methods. However, problems with DNA extractions meant that for all treatments, with exception of PIA, we only obtained qPCR data for cohort II on days 2, 4, 6, 8, 12, 16, 20, 22, 24, 26. For treatment PIA, we obtained qPCR data from cohort I for the same number of days, but samples for this treatment are advanced by a single day, i.e. belong to days 1, 3, 5, 7, 11, 15, 19, 21, 23, 25. Therefore, we analysed the PIA PCR data as part of cohort II, but focused on cumulative parasite densities so that the one-day difference has no (or negligible)

impact on the results. For experiment 2, we analysed parasite densities as quantified by microscopy. Since the aim of this experiment was to test whether the cumulative densities achieved in experiment 1 influenced those achieved in experiment 2, it was necessary to use densities quantified by the same method. Therefore, because we had densities for cohort I, experiment 1, from microscopy we also counted parasites by microscopy for experiment 1 for the relevant cohort II mice.

5.3.4.3. Definition of variables

The analyses of experiment 1 and 2 involve the following variables, which we calculated for every infection:

Cumulative parasite density: total number of parasites produced throughout an infection (i.e. until we stopped the experiment or the host died). We use this as a proxy for parasite performance in an infection, and this takes into account the effect that host mortality has in reducing overall parasite density due to shorter infection length. Note that for data collected by microscopy (cohort 1 in experiment 1 and all of experiment 2) this is the cumulative number of infected RBC, and for data collected by PCR (cohort II in experiment 1) this is the cumulative density of *P. yoelii* genomes (e.g. a single schizont will be counted not as a single parasite, but relative to the number of merozoites that it contains). Although it is possible that our microscopy counts of treatment PIA are higher than they should be due to including *P. chabaudi*, this is unlikely to affect our results because cumulative *P. chabaudi* densities were ~20 times lower than cumulative *P. yoelii* densities (cohort II) and *P. chabaudi* was effectively absent from all PIA infections by day 5 of *P. yoelii* post-infection (Figure S5.2). Indeed, the qPCR results suggest that the only treatment for which the microscopy counts may be significantly biased is treatment M (Figure S5.2). Moreover, we note that we obtain qualitatively similar results for cohort II independently of whether we analyse *P. yoelii* or total (*P. yoelii* + *P. chabaudi*) cumulative parasite densities (data not shown), suggesting that *P. chabaudi* densities do not have a significant impact on the reported results.

Cumulative reticulocyte density: total number of reticulocytes produced throughout an infection (i.e. until we stopped the experiment or the first host died).

Mean reticulocyte proportion: mean proportion of reticulocytes (relative to the total number of RBCs) that were present throughout each infection.

RBC loss: RBC density on day 0 (experiment 1) minus the minimum RBC density measured at any point throughout experiment 1. This quantity was then divided by the day 0 RBC density to account for variation in starting density.

Weight loss: same as for RBC loss, but using weight in the calculations.

Mortality: binary variable coded as 1 if mice survived to the end of experiment 1, and as 0 if not.

5.3.4.4. Analysis of experiments

For experiment 1 (cohort I and II) we first tested whether cumulative parasite density was affected by treatment. When treatment was significant we then tested if treatment levels could be collapsed according to whether our manipulations of the within-host environment led to increased resources (AA and PIA) and/or to increased immunity (AC_S and AC_M). We used AICc and Akaike weights to infer the best combination of treatment levels (Akaike weights reflect the weight of evidence in favour of a model, given the set of models analysed; Burnham and Anderson 2002). Once we identified the best treatment level combination, we used *post hoc* all pairs Tukey tests to identify significant differences between treatment groups. We then explored the effects of immunity and resource availability in more depth by fitting a set of 4 models which included treatment (fitted with the combination of groups inferred above), and variables that are proxies for the frequency (mean reticulocyte proportion) or the density (cumulative reticulocyte density) of available resources, and for heterologous (IgG2a antibody levels to *P. chabaudi*) or homologous (IgG2a antibody levels to *P. yoelii*) immunity. The analyses for experiment 2 followed the same approach but models also included the cumulative parasite density achieved by each infection in experiment 1. We also fitted linear models to the data from experiment 1 to test whether *P. chabaudi* has an effect on the performance of *P. yoelii* in mixed infections and to test which variables explain whether hosts survived their infections. For the latter analysis, we used the entire dataset, without dividing it

into cohorts, and fitted three logistic generalized linear models in which treatment was allowed to interact with weight loss, RBC loss or mean proportion of reticulocytes. We did not use cumulative parasite density as an explanatory variable as this would be confounded by the different methods used to obtain parasite counts. We compared models as described above.

We performed all analysis with R v2.14.0 (R Development Team Core 2011), using log transformed cumulative parasite densities and optical densities (ELISA), to conform to the assumptions of parametric statistics. We minimised models following stepwise deletion of the least significant term and used F-ratio tests to evaluate the change in model deviance until only significant terms remained. Minimal models for each experiment (and cohort) were compared with AICc and Akaike weights. The model(s) with the highest Akaike weight was then used to estimate the amount of variation in cumulative parasite density (or mortality) that is explained by each explanatory variable.

5.4. RESULTS

5.4.1. Validation of treatment effects

First, it was necessary to validate whether the treatments successfully manipulated immunity and/or RBC resources for experiment 1. If the manipulations had the desired effects then at the time of initiating *P. yoelii* infections (day 0, Figure 5.1) we expect the following: (1) PIA and AA should have lower RBC densities and a higher proportion and density of RBCs that are reticulocytes than the other treatments; (2) treatments AC_S and AC_M should have higher levels of *P. chabaudi* specific immunity than the other treatments; (3) the level of *P. yoelii* specific immunity should be similar across all treatments; (4) the *P. chabaudi* pre-infection should generate sufficient immunity to reduce the densities of *P. chabaudi* in the AC_M (or AC_S) group compared to the PIA group (i.e. before this was infected with *P. yoelii*); and (5) the density of *P. chabaudi* in the PIA group should be declining at the moment we inject it with *P. yoeli*.

In support of these expectations, total RBC density (Figure 5.2A) was significantly affected by treatment ($F_{(5,71)} = 191.0$, $p < 0.001$), being lowest for treatments AA and PIA (Tukey test) and this was consistent for both cohorts ($F_{(1,70)} = 0.32$, $p = 0.57$). The proportion (Figure 5.2B) and the density of reticulocytes (not shown) were significantly greater for treatments AA and PIA (Tukey test; treatment effect: proportion: $F_{(5,70)} = 38.1$, $p < 0.001$; density: $F_{(5,70)} = 13.37$, $p < 0.001$). Both the proportion and density of reticulocytes were significantly higher in cohort I than II, but this difference only accounted for ~3 and 6% of the variation (density: $F_{(1,70)} = 8.32$, $p = 0.005$; $2.1x10^8 \pm 4.3x10^6$ [cohort I], $1.7x10^8 \pm 4.7x10^6$ [cohort II]; proportion: $F_{(1,70)} = 8.28$, $p = 0.005$, 0.05 ± 0.002 [cohort I], 0.039 ± 0.002 [cohort II]; mean ± std. error). Treatment groups differed in the level of IgG2a antibodies to *P. chabaudi* ($F_{(5,71)} = 7.48$, $p < 0.001$; Figure 5.2C), but not *P. yoelii* ($F_{(5,71)} = 2.14$, $p = 0.07$; Figure 5.2D), following the same patterns in both cohorts (*P. chabaudi*: $F_{(1,70)} = 0.55$, $p = 0.46$; *P. yoelii*: $F_{(1,70)} = 0.83$, $p = 0.36$). Tukey tests revealed that groups that previously had (AC_S, AC_M) or were carrying (PIA) a *P. chabaudi* infection had higher levels of antibodies to *P. chabaudi* than groups M and S, but not AA. The elevated IgG2a levels in treatment PIA are a consequence of the fact that mice were already being exposed to *P. chabaudi* infection for 10 days when infected with *P. yoelii*. The high antibody levels to *P. chabaudi* for treatment AA were unexpected, as mice in this treatment group had never been infected by *P. chabaudi*, suggesting that PHZ elevates IgG2a responses. However, the elevated antibody levels in AA (cohort I) were transient and had disappeared by day 15 (treatment effect: $F_{(1,59)} = 18.92$, $p < 0.001$; Tukey tests). The anti-*P. chabaudi* responses generated in the AC_M (or AC_S) group strongly reduced *P. chabaudi* densities compared to the PIA group (Figure 5.2E). Finally, the density of *P. chabaudi* in the PIA group was declining by the time *P. yoelii* was added (Figure 5.2F), which explains the elevated reticulocytaemia in this group.

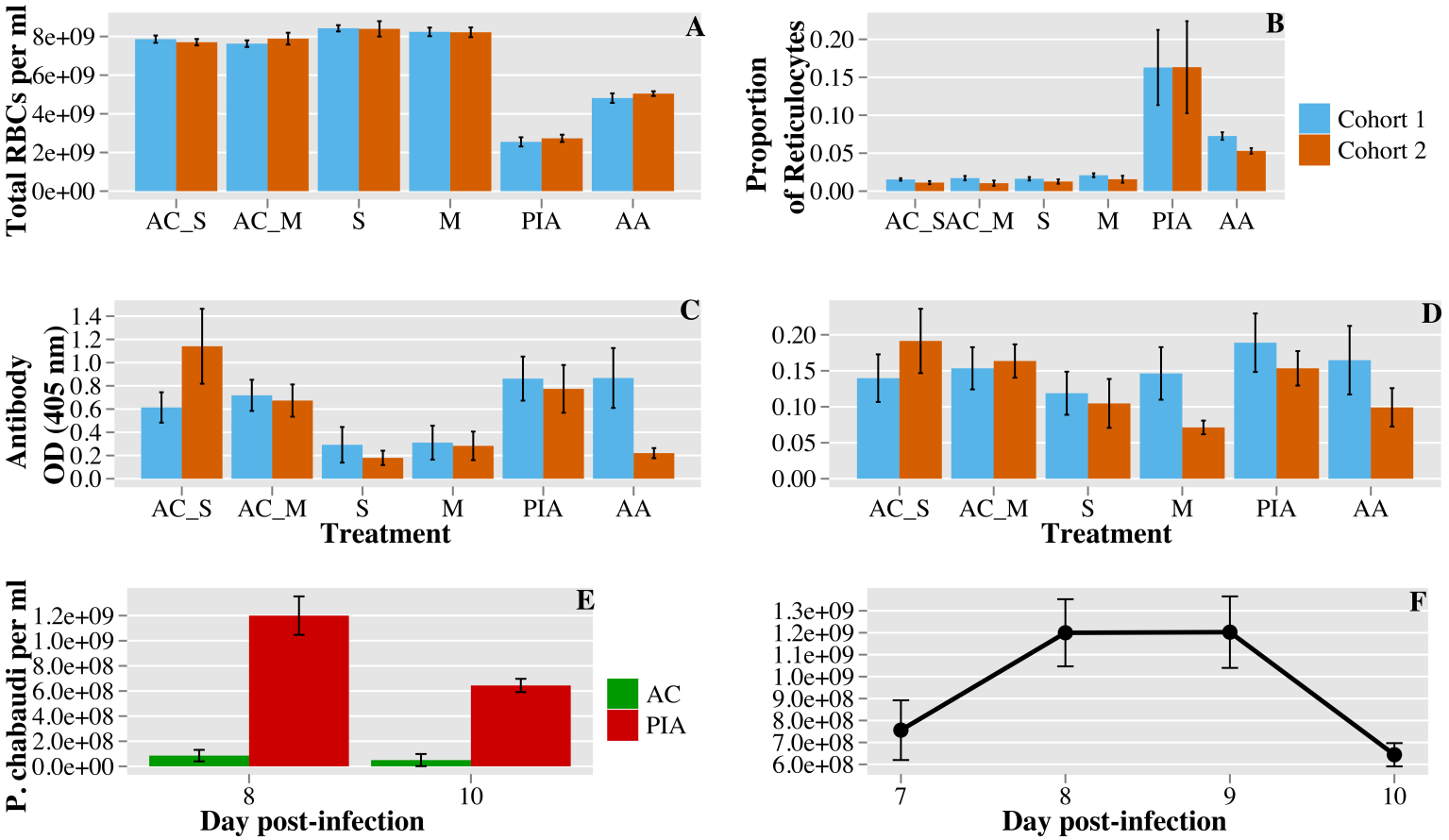

Figure 5.2. Effect of experimental treatments on the within-host environment.

Day 0 means ±SE per treatment and cohort for: Total RBC density (A), Proportion of Reticulocytes (B), (cont.)

Figure 5.2 (cont.) antibody levels to *P. chabaudi* (C) and *P. yoelii* (D), *P. chabaudi* densities at days 8 and 10 post-infection for mice carrying a primary (PIA) or a secondary (AC_S) *P. chabaudi* infection (E), and *P. chabaudi* densities starting to decrease at day 10 for treatment PIA (F). Note, data for E and F were collected before the cohorts allocated.

5.4.2. Experiment 1

5.4.2.1. Effect of treatment

This experiment was designed to test whether *P. chabaudi* could facilitate *P. yoelii* by increasing the availability of *P. yoelii*'s preferred resource (reticulocytes) and whether apparent competition, in the form of immunity to *P. chabaudi,* shapes the dynamics of mixed-species infections. For both cohorts, the density of *P. yoelii* differed significantly across treatment groups (cohort I: $F_{(5,26)} = 7.8$, $p < 0.001$, Figure 5.3A; cohort II: $F_{(5,27)} = 6.9$, $p < 0.001$, Figure 5.4A). We then tested whether treatments could be grouped according to whether the manipulations of the within-host environment increased resource availability (AA and PIA) and/or immunity to *P. chabaudi* (AC_S and AC_M). As shown in Table 5.1, for both cohorts, the model with the highest Akaike weight had the following four treatment levels: AC_S+AC_M (elevated *P. chabaudi* immunity), S, M, and PIA+AA (elevated reticulocytes). For cohort I, all pairs Tukey tests revealed significant differences where cumulative parasite density for PIA+AA was higher than S and AC_S+AC_M, suggesting a strong impact of facilitation via resource availability. Additionally, cumulative parasite density was significantly higher for treatment M than for AC_S+AC_M, but we detect no significant difference between AC_S+AC_M and S, suggesting that the performance of *P. yoelii* was not affected by heterologous immunity (i.e. apparent competition). On the other hand, for cohort II, PIA+AA did not achieve significantly higher densities than treatment S (i.e. no facilitation). However, AC_S+AC_M had significantly lower parasite densities than all other groups, suggesting an effect of heterologous immunity (i.e. apparent competition).

Table 5.1. Akaike weights (and AICc) for models testing the effects of treatment on the cumulative parasite densities observed in experiment 1.

Different treatment levels were collapsed as indicated by a '+' sign and groupings in bold are the most parsimonious, with the 4-level model having the strongest support, for both cohorts.

treatment levels	cohort I	cohort II
6 levels: AC_M; AC_S; S; M; PIA; AA	0.03 (16.7)	0.03 (36.8)
5 levels: **AC_M+AC_S**; S; M; PIA; AA	0.14 (13.9)	0.16 (33.6)
5 levels: AC_M; AC_S; S; M; **PIA+AA**	0.18 (13.4)	0.15 (33.8)
4 levels: **AC_M+AC_S**; S; M; **PIA+AA**	0.64 (10.8)	0.65 (30.8)

5.4.2.2. Effect of resources and immunity

The analysis above for cohort I suggests *P. yoelii* density is strongly facilitated by *P. chabaudi* increasing resource availability. On the other hand, cohort II suggests apparent competition caused by heterologous immunity is important. To examine the effects of immunity and resource availability on cumulative parasite density in more detail, we fitted a set of 4 models to each cohort with explanatory variables that are predictors of the total number (density) or the mean proportion (frequency) of preferred RBC (i.e. reticulocytes) available throughout infections, as well as measures of *P. chabaudi* or *P. yoelii* antibody levels at two different time points (day 0 and 15). The maximal and corresponding minimal models for each cohort are given in Table 5.2. Most of the minimal models contained the following variables: treatment (fitted with 4 levels as described above; 7/8 models), mean proportion or density of reticulocytes (8/8 models) and *P. yoelii* antibody levels on day 15 (3/4 models), with *P. chabaudi* antibody levels never being significant (0/4 models).

Comparing all the minimal models reveals that a single model was overwhelmingly the best, with an Akaike weight ~1, for both cohorts. For cohort I, the best model included treatment ($F_{(3,27)} = 7.66$, $p < 0.001$) and the proportion of reticulocytes ($F_{(1,27)} = 38.64$, $p < 0.001$), explaining 59 and 24% of the variation in parasite density,

respectively. Parasite density correlated positively with the proportion of reticulocytes (Figure 5.3B) and Tukey tests indicate that treatments AC_S+AC_M had significantly lower cumulative parasite densities than all other treatments (Figure 5.3A). These results support those presented in section 5.4.2.1., suggesting that the frequency of reticulocytes can facilitate *P. yoelii*, but indicate that apparent competition occurs in hosts previously exposed to *P. chabaudi*, once the effect of resource availability is accounted for. On the other hand, for cohort II, treatment was non-significant ($F_{(3,27)}$ = 2.37, p = 0.09) and the best model included the proportion of reticulocytes ($F_{(1,30)}$ = 23.07, $p < 0.001$) and day 15 *P. yoelii* antibody levels ($F_{(1,30)}$ = 22.34, $p < 0.001$), explaining 63 and 16% of the variation in parasite density, respectively. As for cohort I, parasite density correlated positively with the proportion of reticulocytes (Figure 5.4B), and negatively with the level of homologous antibody (Figure 5.5). Therefore, cohort II suggests the frequency of reticulocytes can facilitate *P. yoelii* but that homologous (and not heterologous) immunity also has a weak effect.

Table 5.2. Set of maximal models fitted to cumulative parasite density for both cohorts in experiment 1 and the resulting minimal models (page 151).

Akaike weights (and AICc) were used to compare the final set of minimal models. Model in bold had the highest Akaike weight.

Maximal model	cohort I		cohort II	
	minimal model	**Akaike weight (AICc)**	**minimal model**	**Akaike weight (AICc)**
Treatment; Reticulocyte density; *P. yoelii* IgG2a (day 0); *P. yoelii* IgG2a (day 15)	Treatment; Reticulocyte density; *P. yoelii* IgG2a (day 15)	$2.1x10^{-3}$ (-2.19)	Treatment; Reticulocyte density; *P. yoelii* IgG2a (day 15)	$5.07x10^{-3}$ (14.1)
Treatment; Reticulocyte density; *P. chabaudi* IgG2a (day 0); *P. chabaudi* IgG2a (day 15)	Treatment; Reticulocyte density	$2.6x10^{-4}$ (1.92)	Treatment; Reticulocyte density	$4.78x10^{-6}$ (28.1)
Treatment; Proportion of Reticulocytes; *P. yoelii* IgG2a (day 0); *P. yoelii* IgG2a (day 15)	**Treatment; Proportion of Reticulocytes**	**0.998 (-14.55)**	**Proportion of Reticulocytes; *P. yoelii* IgG2a (day 15)**	**0.989 (3.62)**
Treatment; Proportion of Reticulocytes; *P. chabaudi* IgG2a (day 0); *P. chabaudi* IgG2a (day 15)	Treatment; Proportion of Reticulocytes	As above	Treatment; Proportion of Reticulocytes	$5.94x10^{-3}$ (13.8)

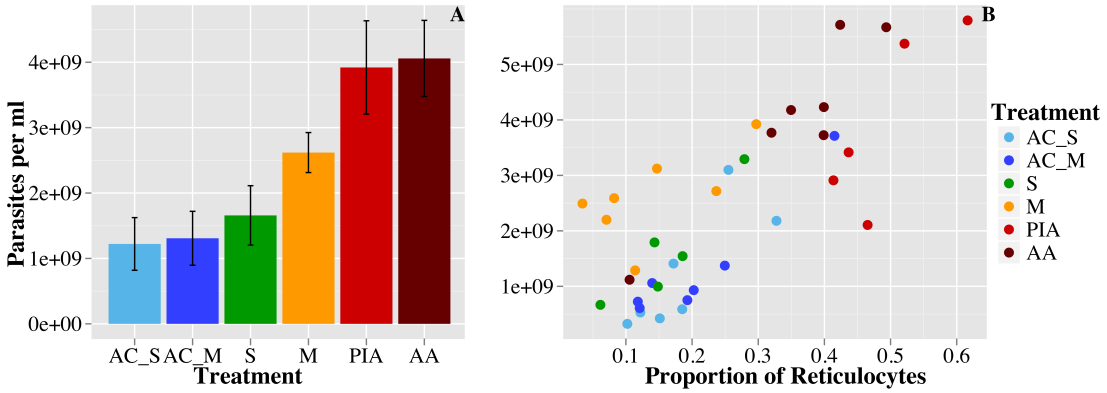

Figure 5.3. Variation in parasite density for cohort I (microscopy), depending on treatment and proportion of reticulocytes (experiment 1).

Mean ± SE cumulative parasite density per treatment (A) and the correlation between parasite density and the proportion of reticulocytes (B).

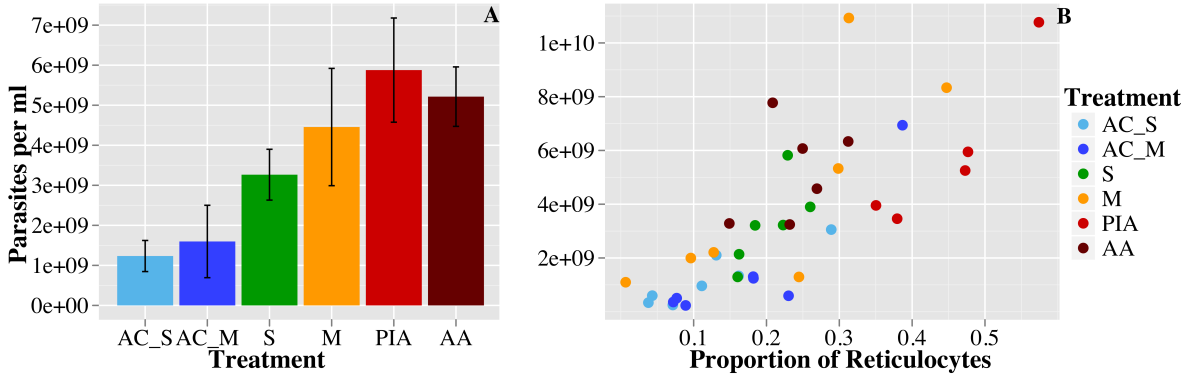

Figure 5.4. Variation in parasite density for cohort II (qPCR), depending on treatment and proportion of reticulocytes (experiment 1).

Mean ± SE cumulative parasite density per treatment (A) and the correlation between parasite density and the proportion of reticulocytes (B).

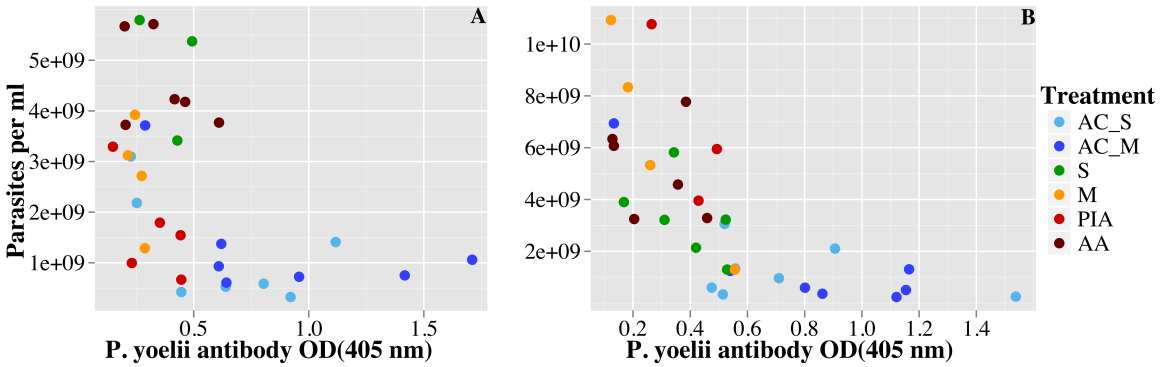

Figure 5.5. Parasite density and day 15 *P. yoelii* antibody levels for experiment 1.

Cohort I (A) and cohort II (B). There was a significant correlation between immunity and parasite density only for cohort II. We present cohort I results for comparison.

5.4.2.3. Density dependent interactions between P. chabaudi and P. yoelii

Because the densities of *P. chabaudi* and *P. yoelii* varied in the 3 mixed infection treatments (AC_M, PIA, M), we used the data from cohort II to ask whether the densities of each species influenced each other. We used data from cohort II only because when examining mixed infections, qPCR counts are more accurate for quantifying each species than the microscopy (particularly for treatment M). We find a significant interaction between species and treatment ($F_{(1,34)} = 19.27, p < 0.001$), with *P. yoelii* densities always being higher than those of *P. chabaudi*, especially in the PIA group (Figure 5.6). Furthermore, the densities of *P. chabaudi* did not significantly correlate with the densities of *P. yoelii* ($F_{(1,15)} = 1.87, p = 0.19$; *P. chabaudi*-treatment interaction: $F_{(2,13)} = 0.0285, p = 0.97$). This suggests the density of *P. chabaudi* itself does not influence *P. yoelii*.

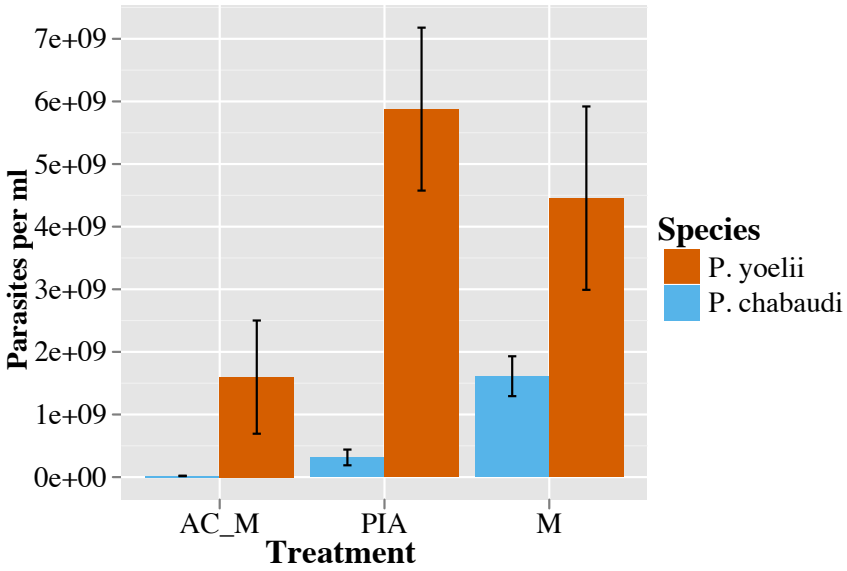

Figure 5.6. Mean±SE *P. chabaudi* and *P. yoelii* density in mixed-species infections for cohort II.

5.4.2.4. Virulence and Mortality

The PIA and M treatments showed extremely exacerbated mortality (~75% of mice died; Figure 5.7A) relative to all other treatments (0-8% mortality), suggesting that without protective immunity, mixed species infections are highly virulent. To test whether this was due to extreme weight loss, RBC loss, or resource abundance for *P. yoelii*, we fitted a series of logistic regression models in which treatment (6 levels) was allowed to interact with: weight loss, RBC loss, and the mean proportion of reticulocytes (the main variable explaining cumulative parasite density). The best model (akaike weight = 0.98) reveals a significant interaction between the mean proportion of reticulocytes and treatment (χ^2_5 = 13.7; p = 0.017), in which the probability of survival generally increases with the mean proportion of reticulocytes for treatments PIA, M and AA and decreases for treatment AC_M (Figure 5.7B). However, results for AA and AC_M should be taken with care, because only a single animal died in those treatments.

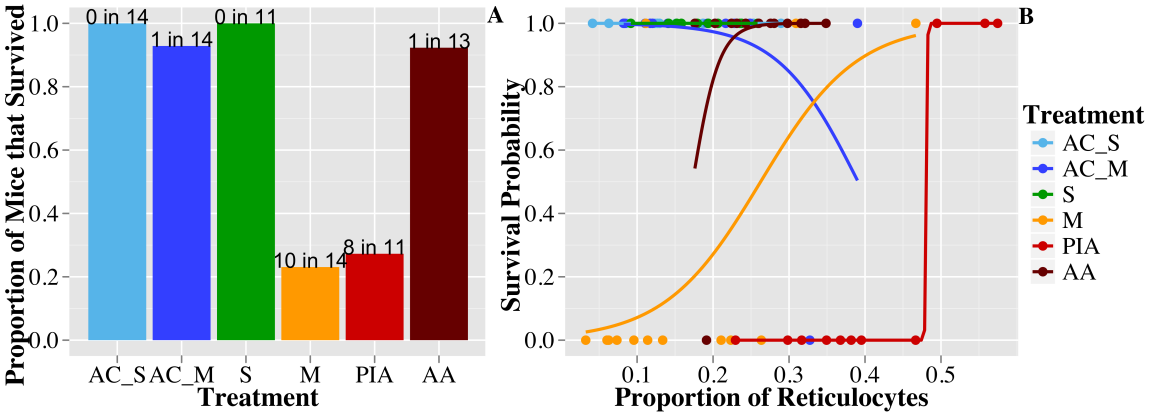

Figure 5.7. Survival in experiment 1.

Proportion of mice, for each treatment, that survived to the end of experiment 1 (A) and predicted relationships between survival probability and proportion of reticulocytes, for all treatment groups (B). In (A), numbers above bars represent the number of mice that died out of the total number of animals in each treatment group.

5.4.3. Experiment 2

This experiment was designed to exploit the variation in *P. yoelii* densities across the different treatments in experiment 1 to test whether there is a trade-off between the densities a species achieves in primary and secondary infections. We focussed on groups AC_S, S, and AA to avoid confounding the analysis with the effects of simultaneous *P. chabaudi* infection. Cumulative parasite density was significantly lower (1-2 orders of magnitude) for experiment 2 than for experiment 1 and the order of ascending density for the different treatments in experiment 2 (AA < S < AC_S) was the reverse of experiment 1 (treatment by experiment interaction: $F_{(1,56)} = 31.49$, $p < 0.001$; Figure 5.8A-B). To test whether the densities achieved in experiment 2 were also shaped by RBC resources and immunity, we fitted a similar set of models to those examined in experiment 1, with an extra variable added to all models: the cumulative parasite density achieved in experiment 1. As for experiment 1, *P. chabaudi* IgG2a levels did not remain in any minimal models, nor did the cumulative parasite density achieved in experiment 1, or day 5 *P. yoelii* IgG2a levels (Table 5.3). Of the minimal models, 2 had similar Akaike

weights (0.36-0.50). These models differ only in the variable used to model resource availability (proportion or density of reticulocytes). We present only the results for the model with proportion of reticulocytes, because in experiment 2, the proportion and density of reticulocytes are very strongly correlated ($R^2 = 0.96$, $F_{(1,28)} = 717.4$, $p < 0.001$) and experiment 1 suggests the frequency of resources is more important than their density. Therefore, the final model included the proportion of reticulocytes ($F_{(1,27)} = 7.11$, $p = 0.013$) and day 0 (for experiment 2) *P. yoelii* antibody levels ($F_{(1,27)} = 5.81$, $p = 0.023$). These variables explained 24 and 14% of the variation in *P. yoelii* density, respectively (Figure 5.8C, D). As for experiment 1, this suggests an important effect of reticulocytes, and a weaker influence of homologous, immunity that develops in response to the challenge of primary infections.

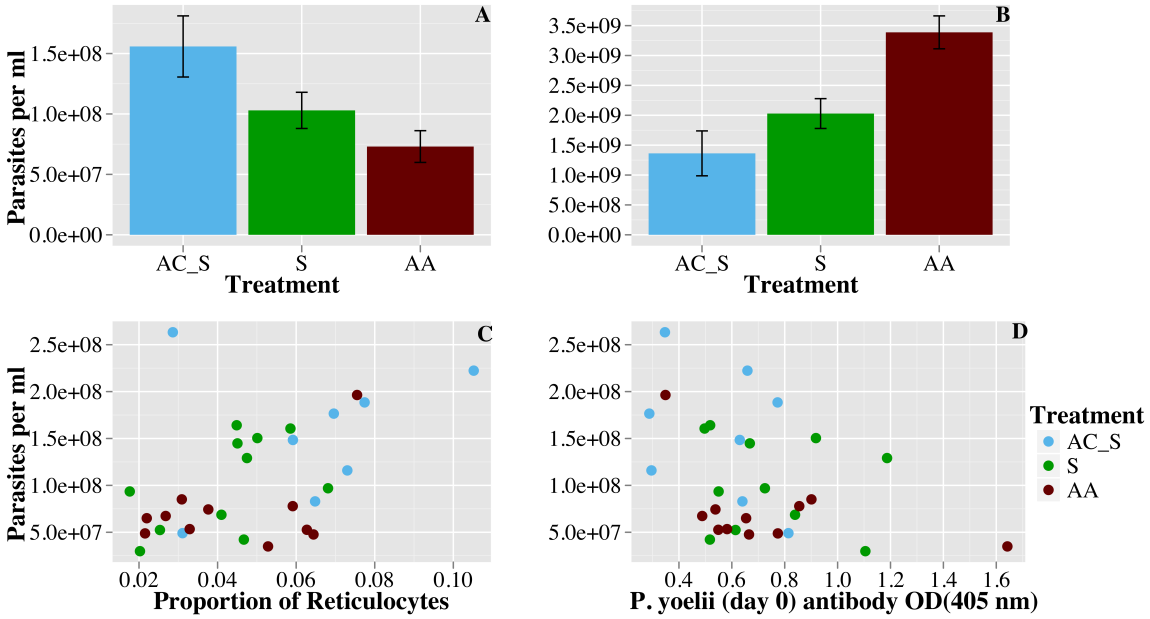

Figure 5.8. Variation in parasite density for experiment 2.

Mean ±SE parasite density per treatment in experiment 2 (A) and for comparison, in experiment 1 (B). Correlation between parasite density, the proportion of reticulocytes (C) and experiment 2 day 0 *P. yoelii* antibody levels (D).

Table 5.3. Set of maximal models fitted to cumulative parasite density for experiment 2 and the resulting minimal models.

Akaike weights (and AICc) were used to compare the final set of minimal models. Models in bold had the highest Akaike weights.

maximal model	minimal model	Akaike weight (AICc)
Treatment; Parasite density in Experiment 1; Reticulocyte density; *P. yoelii* IgG2a (day 0); *P. yoelii* IgG2a (day 5)	**Reticulocyte density; *P. yoelii* IgG2a (day 0)**	**0.36 (0.41)**
Treatment; Parasite density in Experiment 1; Reticulocyte density; *P. chabaudi* IgG2a (day 0); *P. chabaudi* IgG2a (day 5)	Treatment;	0.04 (3.68)
Treatment; Parasite density in Experiment 1; Proportion of Reticulocytes; *P. yoelii* IgG2a (day 0); *P. yoelii* IgG2a (day 5)	**Proportion of Reticulocytes; *P. yoelii* IgG2a (day 0)**	**0.50 (-0.38)**
Treatment; Parasite density in Experiment 1; Proportion of Reticulocytes; *P. chabaudi* IgG2a (day 0); *P. chabaudi* IgG2a (day 5)	Proportion of Reticulocytes	0.10 (1.75)

5.5. DISCUSSION

We undertook several experiments, involving the manipulation of the within-host environment, to test how apparent competition, facilitation, and infection history affect parasite performance. First, we show that in naïve mice, mixed-species infections can enhance (i.e. facilitate) *P. yoelii* relative to single infections and that this is driven by reticulocyte (resource) availability. Specifically, our data suggest the frequency (proportion) of reticulocytes is more important than their number (density). Second, *P. yoelii*'s performance in mice that previously had a *P. chabaudi* infection is reduced relative to single *P. yoelii* infections. Whilst this demonstrates apparent competition occurs, we note that we did not detect an effect of heterologous immunity as measured by IgG2a and that treatments AC_M and AC_S were not significantly different. Third, *P. chabaudi* parasite densities do not directly affect *P. yoelii* replication. Fourth, the mortality rate of naïve hosts is highly exacerbated in mixed- relative to single-species infections and survival probability is related to the proportion of reticulocytes. Finally, in mice that recover from a *P. yoelii* infection, *P. yoelii* densities in secondary infections are 1-2 orders of magnitude lower. Additionally, the reduction in parasite density in secondary infections depends on the density achieved during a primary infection, suggesting a trade-off between current and future performance. Below we discuss each set of results, their implications, and possible methodological limitations of our experiments.

5.5.1. Resource availability and facilitation

P. yoelii's performance in the PIA treatment is either enhanced (cohort I) or not different (cohort II) from its performance in single infections (S) revealing that *P. chabaudi* can facilitate *P. yoelii*. On the other hand, while we detect facilitation in treatment PIA, we do not observe the same for treatment M. This indicates that the order of infection can influence the type of interactions between parasites in mixed-species infections, which is also known to occur in mixed-genotype infections (de Roode et al. 2005).

In agreement with theoretical models predicting facilitation of *P. vivax* by *P. falciparum* (McQueen and McKenzie 2006), it is likely that the facilitation of *P. yoelii* by *P. chabaudi* results from an increase in reticulocyte availability, as the mean proportion of reticulocytes is the main continuous variable explaining *P. yoelii* density in our statistical models and this was significantly higher for PIA than for all other treatments at the start of the infections (Figure 5.2B). That the mean proportion rather than the absolute density of reticulocytes strongly affects *P. yoelii*'s performance suggests a merozoite's ability to find and invade RBCs is a random process whose success depends on the frequency of contact with reticulocytes (relative to normocytes).

Whilst facilitation has been shown to occur between different conspecific genotypes of *P. chabaudi*, this is uncommon, occurs only during the chronic phase of infections, and is most likely mediated by strain-specific immunity (De Roode et al. 2004a; De Roode et al. 2004b; Bell et al. 2006; Wargo et al. 2007b). Here we show that facilitation can also occur during the acute phase of infection and be mediated by resource availability. Interestingly, this occurs despite the fact that mice in the PIA group are controlling the first peak of a *P. chabaudi* infection (Figure 5.2F), for which the innate immune response, that should affect all parasites equally, is thought to be essential (Stevenson and Riley 2004).

5.5.2. Apparent competition, heterologous and homologous immunity

In general, our analyses show that *P. yoelii*'s performance was adversely affected by a previous *P. chabaudi* infection, suggesting that apparent competition mediated by heterologous immunity occurs (but has a weaker effect than facilitation). However, if this is the case then why did we not detect an effect of *P. chabaudi* IgG2a levels on *P. yoelii*? A lack of effect of heterologous immunity is relatively common, including for the parasite species we used (McColm and Dalton 1983; Jarra and Brown 1985). There are several non-mutually exclusive explanations. First, the assay we used for *P. chabaudi* measures IgG2a antibodies against merozoite surface protein 1 (MSP1). Antibodies to this protein are a major determinant of protection in malaria infections (O'Donnell et al. 2001; Burns et al. 2004), however there is also some evidence that the immunity

generated is strain-specific (Pattaradilokrat et al. 2007), which could prevent us from detecting an effect of heterologous immunity. Second, homologous immunity (measured as total IgG2a against *P. yoelii's* crude lysate) weakly correlates with *P. yoelii*'s performance in cohort II (experiment 1; Figure 5.5B) and there was a significant correlation between *P. yoelii* and *P. chabaudi* antibody levels on day 15 (Figure S5.4). Third, on day 15, antibody levels to *P. yoelii* are higher for AC_M+AC_S than for the S or M treatments. These observations suggest that a previous *P. chabaudi* infection enables animals to acquire a stronger response against *P. yoelii,* or leads to heterologous immunity that we were unable to measure.

5.5.3. P. yoelii-P. chabaudi interactions

Our results suggest that the presence of *P. chabaudi* did not affect the performance of *P. yoelii*. Specifically, *P. yoelii*'s performance was similar in the presence or absence of *P. chabaudi* in naïve hosts (M vs S), hosts with elevated immunity to *P. chabaudi* (AC_M vs AC_S) and in hosts with increased reticulocyte availability (PIA vs AA). *P. yoelii*'s cumulative parasite densities were always higher than those of *P. chabaudi* in mixed-infections and we find no correlation between the parasite densities of the two species. This suggests that *P. yoelii* and *P. chabaudi* may present discrete challenges to the host in terms of the resources they consume. Given that *P. chabaudi* infects a broader range of RBCs than *P. yoelii*, these results contradict the normally held notion that parasites with a broader RBC age preference will be competitively superior (Antia et al. 2008). Therefore future theoretical models of within-host competition should take into account not only the range of RBC ages that are susceptible to infection, but also how RBC dynamics affect parasite proliferation, and in turn, how this affects the distribution of RBCs across different age classes.

5.5.4. Virulence and mortality

Although an often ignored fact for practical reasons, virulence is not a trait solely of hosts or parasites, rather it is a compound trait that depends on the host and all the parasites co-infecting it (Alizon and Van Baalen 2008). Our results, in which mortality was greatly

exacerbated in treatments M and PIA are a good illustration of this. Whilst mice in these treatments harboured high density infections, similar parasite densities were observed in the AA treatment where only a single fatality occurred. Thus, parasite density *per se* does not seem to be a major determinant of mortality. Moreover, contrary to previous work (Mackinnon and Read 2004), our results show that survival probability is not correlated to two virulence proxies typically used in rodent malaria studies (RBC density and weight loss). We show that for treatments PIA and M, survival probability increases with the mean proportion of reticulocytes. Given that this variable is also strongly and positively correlated with cumulative parasite density, we suggest that the balance between the production and destruction of RBCs is particularly fragile in mixed-species infections. Indeed, during malaria infections multiple factors influence RBC availability (e.g. dyserythropoiesis, bystander killing) and it has been shown that in single *P. yoelii* or *P. chabaudi* infections, the host can benefit from a decrease or an increase in reticulocyte production, respectively (Chang and Stevenson 2004).

5.5.5. Trade-off in current versus future performance

The results from experiment 2 show that across the three tested treatments (S, AC_S and AA), *P. yoelii*'s performance is significantly impaired in a secondary challenge and that the order in which cumulative parasite density (across treatments) increases in experiment 1 is reversed for experiment 2. This suggests that a trade-off between parasite performance in a primary versus a secondary infection. Given that malaria transmission will tend to be local, if such an effect occurs in the wild, this could represent an additional selective pressure for parasites to modulate growth rate to find a compromise between the rate of replication and the duration of infection. Furthermore, despite parasite densities in the secondary challenge being considerably lower than the primary infection we still find that reticulocytes explain the most variation in *P. yoelii* density. However, we note that the variation in *P. yoelii*'s performance that we can explain for experiment 2 is substantially lower than for experiment 1, suggesting that additional factors operate in experiment 2. These could include other classes of antibodies, such as IgG1 or IgM, which can also mediate protection (Mota et al. 1998).

5.5.6. Implications and future directions

Our results have two main implications for our understanding of the biology of mixed infections. Firstly, whilst it has for long been held that *P. vivax* reduces the pathology caused by *P. falciparum*, two recent studies suggest that this is not the case. Studies by (Genton et al. 2008; Tjitra et al. 2008) in two different endemic areas show that the proportion of patients with severe malaria was higher (across all age classes) for patients with mixed-infections than for patients with single infections of *P. falciparum* or *P. vivax*. Moreover, this difference was particularly apparent in young children (< 5 years old) and anaemia was one of the main factors contributing to disease. Our work also shows highly exacerbated mortality in mixed infections of naïve hosts and indicates that *P. yoelii* parasites are able to fuel their growth with the excess reticulocytes that are produced in response to a *P. chabaudi* infection. This pattern could explain the oscillations observed in neurosyphilis patients, where mixed *P. falciparum* and *P. vivax* alternated dominance in mixed infections (Mason and McKenzie 1999; Zimmerman et al. 2004).

Secondly, the impact of mixed-species infections on parasite fitness is still unclear, with different studies reporting both positive and negative impacts. Our results, suggest that prior infection with a generalist or a species that prefers mature RBC can positively affect an incoming species of malaria parasite that prefers reticulocytes. However this is disadvantageous for the prior resident because by facilitating the incomer, its supply of older RBC is cut off (McQueen and McKenzie 2004). Determining how these effects quantitatively influence the in-host survival of co-infecting species requires detailed examination of infection dynamics because small shifts in the details of the order of infection and the erythropoesis response could substantially alter patterns. Understanding the impact of mixed species malaria infections also requires testing how between host transmission is affected. For example, assuming within-host growth is positively correlated to between host transmission, reticulocyte preferring parasites achieve higher transmission from mixed infections. However, if, as data suggest, hosts are at higher risk of mortality, there may be a trade-off with the duration of transmission.

Furthermore, experiments with *P. chabaudi* reveal that conspecific genotypes reduce investment in transmission stages when in competition (Pollitt et al. 2011). Therefore, the lifetime transmission success of a species in a mixed infection will depend on the impact of facilitation and suppression, whether parasite phenotypic plasticity can enhance or ameliorate the effects of facilitation and suppression, and the consequences of mixed infections for host longevity.

5.6. APPENDIX

Figure S5.1. The infection dynamics of *P. yoelii* in cohort II (Experiment 1).

Figure S5.2. The infection dynamics of *P. chabaudi* in cohort II (Experiment 1) for treatments M (yellow) and PIA (green).

Figure S5.3. The infection dynamics of *P. chabaudi* in cohort II (Experiment 1) for treatment AC_M.

Figure S5.4. Correlation between day 15 antibody levels for *P. yoelii* and *P. chabaudi*.

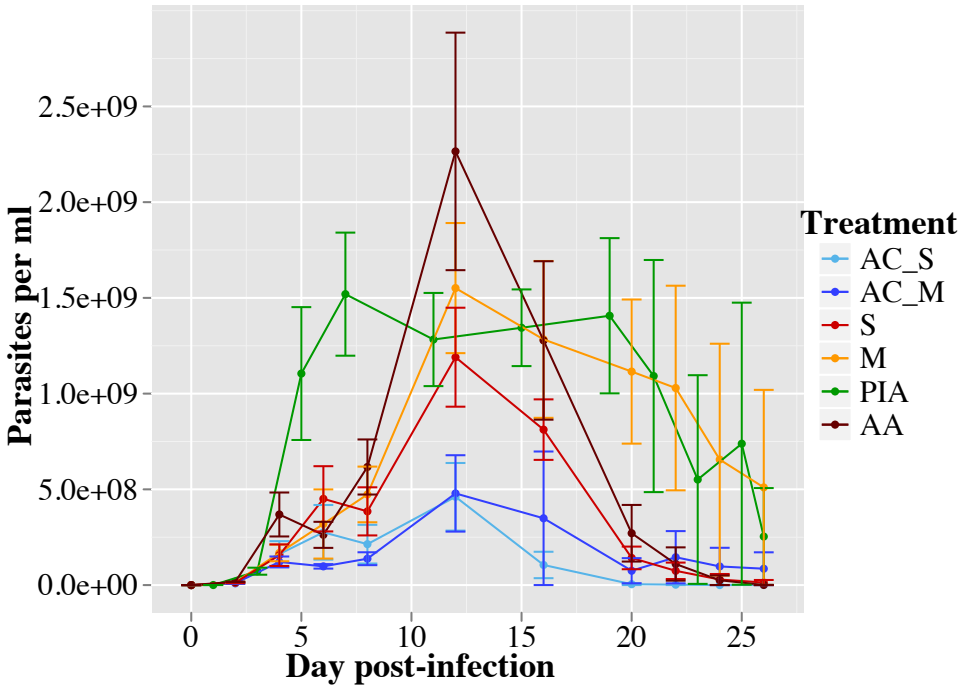

Figure S5.1. The infection dynamics of *P. yoelii* in cohort II (Experiment 1). Mean ± SE parasites per ml per day.

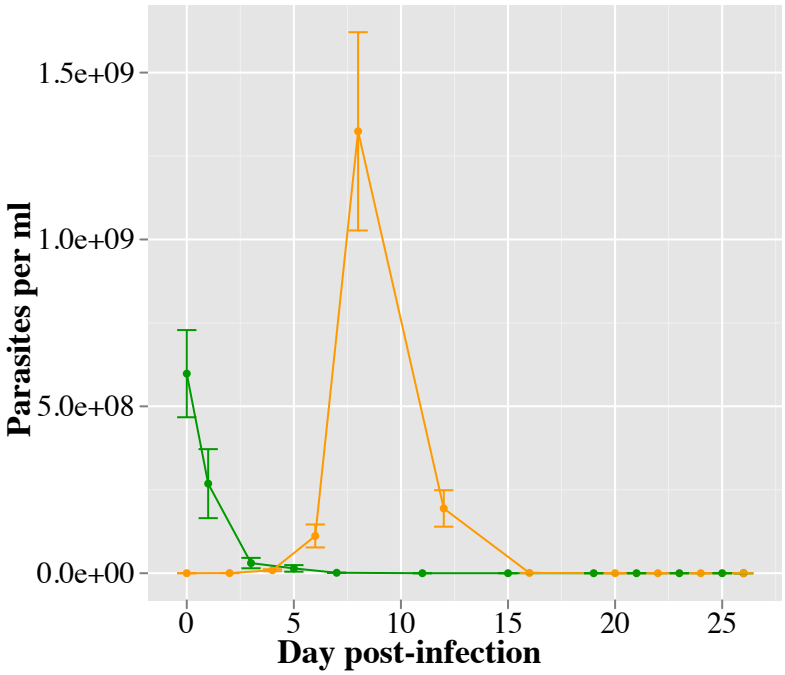

Figure S5.2. The infection dynamics of *P. chabaudi* in cohort II (Experiment 1) for treatments M (yellow) and PIA (green). Mean ± SE parasites per ml per day.

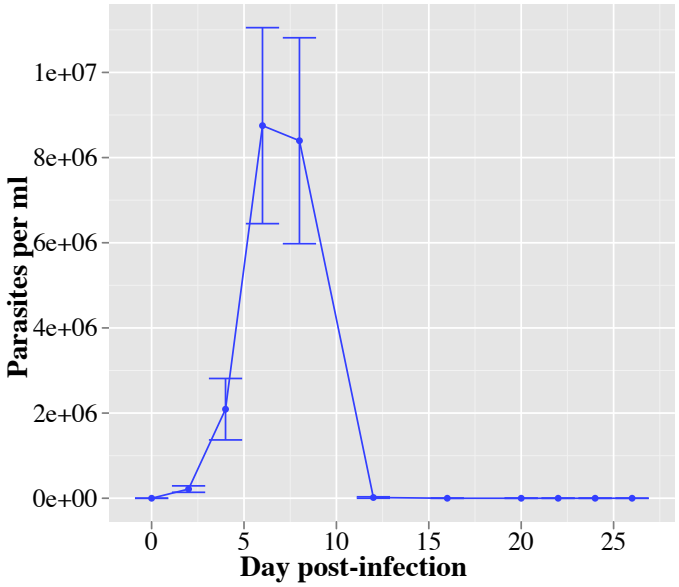

Figure S5.3. The infection dynamics of *P. chabaudi* in cohort II (Experiment 1) for treatment AC_M. Mean ± SE parasites per ml per day. Note that y axis scale is ~2 orders of magnitude lower than in Figure S5.2.

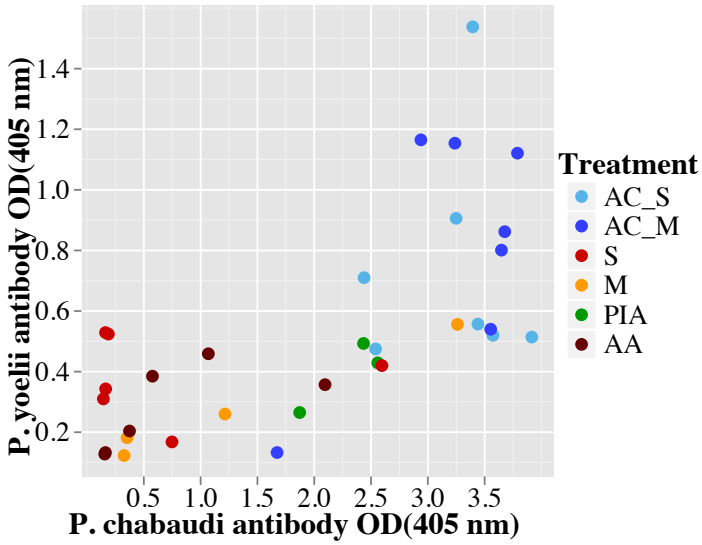

Figure S5.4. Correlation between day 15 antibody levels for *P. yoelii* and *P. chabaudi* for cohort II (experiment 1). There is a significant interaction between treatment and *P. chabaudi* antibody levels ($F_{(5,21)} = 4.41$; $p = 0.007$). Similar results were obtained for cohort I.

6. General Discussion

The study of parasite biology has mostly been undertaken within the remit of reductionist disciplines (cellular and molecular biology, immunology, etc). This research has provided information on a variety of biological problems, particularly those concerning gene function, but in the search for drug/vaccine targets, parasite behaviours and the ecological interactions that occur within-hosts have been neglected. On the other hand, biological processes that occur at the molecular, cellular, and physiological levels of parasites have often been viewed as "details" by epidemiologists and evolutionary biologists, who try to understand host and parasite population level patterns in virulence, transmission, and recovery (Mideo et al. *in press*). Due to this disconnect, there is little understanding of how parasites maximise fitness across scales of biological organization (i.e. how they live in cells, hosts, and vectors). However, given that the within-host processes determine parasite transmission from the host, what happens during infections clearly influences between-host processes and will therefore influence population level traits. There is now increasing recognition that integration across within- and between-host processes is necessary, and that this requires interdisciplinary approaches (Anderson 1994; Restif 2009; Mideo et al. *in press*). In order for this integration to be fruitful, a better understanding of the whole-organism biology of parasites is needed, especially how basic processes in the host shape transmission success (including how parasites interact with hosts and other co-infecting parasites). In this thesis I have studied malaria parasites and focused mainly on the interface between how within-host processes shape transmission to the vector. Below I summarize the results for each chapter and highlight areas for future work.

6.1. PHYLOGENETICS

In chapter 2, I apply recently developed species delimitation methods to rodent malaria parasites to obtain a phylogeny and provide the first estimates of divergence time, polymorphism and population sizes. My key finding is that my analyses

suggest that all rodent malaria subspecies can be considered species. While the application of species delimitation methods to rodent malaria parasites can be criticized (because phylogenetic and geographical effects cannot be separated for this group of parasites), two different methods suggest that all subspecies could be considered species and in particular, show that most of *P. vinckei*'s subspecies diverged either earlier or within the same time frame as *P. berghei* and *P. yoelii*, supporting the conclusion that these are different species. These results are important because the definition of species, despite its many problems, is central for the interpretation of multiple biological processes (Whelan 2011). Importantly, phenotypic traits that are normally measured from one or a few genotypes of a single subspecies of rodent *Plasmodium* are often generalized to be representative of what occurs at the (named) species level, which can lead to confusion. For example, it is often stated that *P. vinckei* is a synchronous parasite (i.e. parasites in an asexually replicating cohort progress through the cell cycle in sync with each other, simultaneously busting at schizogony) but recent work contradicts this (A. O'Donnell, personal communication). This may be due to studies using different "sub" species. More generally, the delimitation of species allows the prediction of which groups of genotypes are reproductively isolated (Whelan 2011). This can help understand patterns of gene flow, which will impact on the epidemiological dynamics of traits underpinning transmission, virulence, and drug resistance (Lymbery and Thompson 2012) .

The development of new sequencing technologies and phylogenetic methods is also starting to contribute more broadly towards the understanding of parasite biology (Archie et al. 2009). Firstly, in rapidly evolving parasites (e.g. HIV), where evolutionary and ecological processes occur at similar time scales, phylogenetic methods can be used to identify transmission pathways across a variety of temporal and spatial scales, infer ecological interactions such as competition, top-down and bottom-up regulation, and identify the origin of epidemics (Archie et al. 2009). Secondly, for slowly evolving parasites (e.g. malaria, parasitic nematodes), phylogenetic methods can be applied to study processes such as host-switches and the spread of drug resistance (Archie et al. 2009). Finally, very recently, methods to

estimate correlations between phenotypic traits and infer ancestral trait states have been implemented in the popular software package BEAST. This may be an interesting tool for experimentalists to estimate possible historical constraints and trade-offs between pairs of traits and for identifying changes in trait states through evolutionary timescales (Drummond et al. 2012).

6.2. HYBRIDISATION AND SPECIES RECOGNITION

In chapter 3, I show that hybridisation between two rodent malaria parasites (*P. berghei* and *P. yoelii*) can occur, but only occurs at high levels when one of two (P230 or P48/45) proteins are absent from the surface of female gametes. This indicates that P230 and P48/45 are gamete recognition proteins. Moreover, I find that these proteins and a possible interaction partner (P47) are fast evolving, a feature that is often observed in gamete recognition proteins in other taxa (Swanson and Vacquier 2002). Finally, I show that asymmetric reproductive interference occurs, with the fertilisation success of *P. berghei* being reduced in the presence of *P. yoelii*, but not vice-versa.

The mating ecology of malaria parasites has often been difficult to study, both at the whole organism and at the molecular level, due to two main reasons: (i) gametogenesis and fertilisation occur within a short time frame and gametes (especially males) are extremely short-lived (Sinden and Croll 1975; Janse et al. 1986; Alano and Carter 1990; Talman et al. 2004); and (ii) it is difficult to separate male and female gametocytes/gametes (Khan et al. 2005). The latter problem is more significant but can now be overcome by using genetically modified parasites that either express different fluorescent proteins in male and female gametocytes/gametes (so they can be separated by fluorescence activated cell sorting) or that have been knocked-out for genes that make male or female gametes unviable (similar to those I used in chapters 2 and 3) (van Dijk et al. 2001; van Schaijk et al. 2006; Ponzi et al. 2009; van Dijk et al. 2010). Using these tools, several important questions can now be asked and I outline some of these below.

I showed that while hybridisation occurs at high levels in the absence of P230 or

P48/45 from the surface of females, mating was still non-random. I suggested two possible reasons for this. First, there could be other proteins that are involved in gamete recognition, such as P47 or the LAP/CCp proteins. However, given that we still know relatively little about the protein composition of the surface of male and female gametes, it is also possible that there are other proteins involved that remain unidentified. Second, there could be species-specific chemotatic signals that allow male gametes to preferentially move towards conspecific females – a common process in many externally fertilizing species (Vacquier 1998). If so, my asymmetric reproductive interference results suggest that such signals may differ in their attractiveness to males of other species. Given that it is now possible to separate male and female gametes, it should also be possible to test whether chemotaxis exists among malaria gametes. If chemotaxis occurs and has an important fitness benefit, disrupting this could prevent/reduce fertilisation and therefore transmission.

The identification of P230 and P48/45 as fast evolving gamete recognition proteins raises the question of whether gamete compatibility correlates with the level of pairwise divergence in these proteins. (Zigler et al. 2005) found evidence of such a correlation in pairs of congeneric sea urchin species, for which divergence in *bindin* (a sperm protein that is involved in recognition) at non-synonymous sites negatively correlates with gamete compatibility, whilst no correlation is obtained for synonymous sites or for divergence at mitochondrial loci. Obtaining similar data for malaria parasites could be done using similar approaches to those I present in chapter 3, providing parasite lines expressing different fluorescent proteins are available for a variety of *Plasmodium* species. Importantly, if such a correlation exists and can be expanded to mating between conspecifics, it could be an interesting tool for predicting patterns of gene flow.

It has been suggested that the geographical divergence observed in P230 in *P. falciparum* could be due to local adaptation to different mosquito populations (D. Kwiatkowski, personal communication) or to host immune responses (van Dijk et al. 2010). P230 (and P48/45) can trigger potent transmission-blocking immune responses but interactions between P230 and the vector are yet to be demonstrated.

However, in chapter 3, I suggested there could be other explanations for this rapid evolution: sexual conflict (mediated by polyspermy) or reinforcement. Testing for reinforcement involves comparing the level of reproductive isolation of focal parasite populations that are in sympatry or in allopatry with heterospecificcs (Nosil et al. 2003). This is not possible for rodent malaria parasites because each of the available species/subspecies represents a single parasite population (according to the samples currently available). On the other hand, testing whether polyspermy occurs would simply involve assaying whether the proportion of viable zygotes decreases with male gamete density (for a given density of female gametes) and whether this is due to multiple fertilisations (which could be observed by staining the DNA of zygotes; e.g. as in Reininger et al. 2005). Besides revealing whether sexual conflict can occur in malaria parasites, such experiments would provide information for whether female gametes can prevent polyspermy. Several organisms have effective systems that block polyspermy (Jaffe and Cross 1986; Sun 2003), but it is unknown whether such blocks exist in malaria parasites.

Finally, asymmetric reproductive interference could have important impacts on our ability to understand the transmission dynamics of malaria parasites, as it will change the predictions that can be made from observations at the within-host level. While I cannot clearly identify what mediates reproductive interference, I suggested several possibilities in chapter 3. In addition to chemotaxis cues (mentioned above) one other possibility is allelopathy (a chemical-based form of interference competition). While allelopathy has not been shown for malaria parasites, I suggest that the conditions within the vector are more suitable for the evolution of allelopathy than the conditions within the host. While in the host, parasites are contained in a large blood volume and will be circulating in the blood stream (except during sequestration). On the other hand, in the vector, parasites will be contained in <2μl of concentrated blood, making the scale of interactions more local than in the host. Local interactions have been shown to be essential for promoting the evolution of allelopathy in bacteria (Chao and Levin 1981).

6.3. SEX ALLOCATION

While the evolutionary biology literature contains many tests of the predictions of sex allocation theory, its assumptions are less well tested. In chapter 4, I explore the effects of two important innate immune factors (TNF-α and reactive nitrogen species) on gametogenesis and fertility of male and female gametocytes/gametes. These experiments provide the first direct test of the assumption that immunity affects the fertility of males more than females. I show that while the fertility of both males and females is equally affected, males are affected during gametogenesis and females are mostly affected through gamete dysfunction, which is in agreement with the assumptions of theory.

I show that immunity can have complex, stage and sex-specific effects, from: reducing gamete production, to generating gamete dysfunction (i.e. gametes can mate but zygotes fail to develop), to affecting the viability of zygotes. Together with João Alpedrinha and Andy Gardner (Oxford University), we generated new theory to predict the evolutionary trajectories of parasite sex ratio strategies, given the range of effects caused by immunity. Our results show that if immunity reduces gametocyte/gamete development, sex ratio adjustment can bring fitness returns. On the other hand, there are no fitness benefits of changing sex ratio if immunity leads to gamete dysfunction or directly reduces zygote development. Given the drive to develop medical interventions that prevent transmission by blocking parasite mating (Carter 2001), these results can have important implications. For example, interventions targeting gamete dysfunction or zygote development will be more "evolution-proof" than current efforts to target sex-specific proteins that prevent fertilisation.

Sex allocation theory has been successful at explaining the sex ratios of malaria parasites and predicting how they should be adjusted in response to a variety of environmental factors. Malaria parasites can facultatively adjust their sex ratios and the extent of the plastic response suggests that parasites can match their sex ratio strategies to a range of environmental conditions, provided they can identify reliable

cues for such an adjustment (e.g. (Reece et al. 2008) showed that different genotypes of *P. chabaudi* adjust their sex ratios between ~0.05 to 0.65 and (Mitri et al. 2009) showed a sex ratio adjustment between 0.04-0.76 for a line of *P. falciparum*). This suggests that parasites may be able to adaptively adjust their sex ratios in response to novel environmental challenges (e.g. a transmission blocking vaccine that generates a sex-specific immune response) without requiring recombination or beneficial mutations to evolve new fixed strategies.

A key assumption of adaptive plastic sex ratio adjustment is that malaria parasites can detect and respond to in-host cues that reliably predict how environmental factors will affect male and female gametocytes/gametes in the mosquito vector (West et al. 2001; Paul et al. 2002a; West et al. 2002; Gardner et al. 2003; West 2010). The main environmental factors to which parasites have been predicted to respond are immunity, anaemia and the genetic diversity of infections (Paul et al. 2000; West et al. 2001; Paul et al. 2002a; West et al. 2002; Gardner et al. 2003; Reece et al. 2005). Therefore, identifying the environmental cues and molecular mechanisms parasites use in their sex ratio decisions is now required. The only molecular cue that has been suggested up until now is the hormone erythropoietin (EPO) (Paul et al. 2000; Paul et al. 2002a). Specifically, it has been shown that in response to EPO, *P. vinckei* and *P. gallinaceum* (an avian parasite) increase their sex ratios (Paul et al. 2000; Paul et al. 2002a; Reece et al. 2005). EPO normally increases 3-4 days before an acute increase in the number of reticulocytes in the bloodstream, which occurs during anaemia (Jelkmann 2001). As anaemia and immunity increase at similar timescales during infections, it has been suggested that EPO could be a cue for either of these environmental factors, both of which are predicted to decrease the likelihood of male gametes finding female gametes (Paul et al. 2000; West et al. 2001; Paul et al. 2002a). It is also not clear from the timing of responses to EPO, whether parasites are responding directly to EPO or the resulting increase in reticulocytes (Reece et al. 2005). Identifying the cues that trigger sex ratio changes that are adaptive to malaria parasites is important as it could lead to new interventions that aim to induce parasites to make suboptimal decisions (Reece et al. 2009; Mideo and Reece 2012). In this regard, an important future direction will be to

examine the fitness benefits that parasites obtain from adjusting their sex ratio.

6.4. MIXED-SPECIES INFECTIONS

In chapter 5, I show that co-infection with *P. chabaudi* can boost *P. yoelii* parasite densities. This facilitation is mediated by elevated resource availability. On the other hand, to a lesser extent, apparent competition also operates. Additionally, I show that the development of homologous immunity against *P. yoelii* leads to a trade-off between current and future performance, such that *P. yoelii* density in a primary infection is negatively correlated to that achieved in a secondary infection. Finally, I show that mixed-species infections (*P. yoelii* plus *P. chabaudi*) lead to greatly exacerbated virulence, beyond that predicted solely by infection intensity.

Facilitation and apparent competition could have an important role in the transmission dynamics of malaria parasites and could affect the likelihood of observing mixed-species infections in nature. A variety of studies have aimed to understand whether mixed-infections are more or less common than expected if the different species had no positive or negative effects on each other (Richie 1988; McKenzie and Bossert 1997, 1999; Howard et al. 2001). However, at the moment it seems impossible to draw any general conclusions because the type of association varies across spatial and temporal scales and also depends on the specific species involved. Nevertheless, two studies by Mckenzie and Bossert suggest a deficit of mixed-species infections in areas where *P. falciparum*, *P. vivax* and *P. malariae* co-occur and a surplus in areas where *P. falciparum*, *P. malariae* and *P. ovale* are present (McKenzie and Bossert 1997, 1999). Given my results, it is likely that these observations are highly influenced by within-host processes. Therefore it would be important to dissect which immunological, haematological and nutritional factors may affect the strength and type of association between different *Plasmodium* species.

I reveal an important role for RBC age preference in mediating interactions between different parasite species. RBC age preference is normally treated as a fixed parasite trait, but it is also known that this can be affected by immunity. For example, *P.*

chabaudi infects reticulocytes at higher rates in CD4 T cell depleted mice than in normal mice (Taylor-Robinson and Phillips 1994). This suggests that, depending on the host immune status, interactions between parasite species could flip between having negative or positive effects on parasites and hosts. This could be particularly relevant in areas where malaria parasites coexist with other parasites that are known to alter immune profiles (e.g. HIV or parasitic helminths). Moreover, while RBC age preference is normally analysed as a binary trait (reticulocytes vs normocytes), this is likely an artifact of the available methods for quantitatively aging RBCs. In the future it will be important to develop methods that can reveal RBC age as a continuous trait so that it is possible to estimate the range of RBC ages that parasites can infect. This will enable predictions to be more easily made about whether positive (facilitation) or negative (competition) interactions will occur among different con-specific genotypes as well as species (Antia et al. 2008).

I observed strongly exacerbated virulence and higher *P. yoelii* densities in mixed-species infections of naïve hosts. This is in agreement with recent reports of high morbidity and mortality in mixed-species infections between *P. falciparum* and *P. vivax* (Genton et al. 2008; Tjitra et al. 2008; which share similar RBC preference to *P. yoelii* and *P. chabaudi*). Therefore it is important to understand how virulence evolves in response to mixed-species infections. Whether mixed-species infections will select for higher or lower virulence will depend on several factors: (i) the frequency of mixed-infections; (ii) the benefits of higher parasite densities for transmission; (iii) the costs of a shorter infection length (due to host mortality); (iv) whether parasites can facultatively respond to the presence of competitors by changing their growth rates; (v) whether the immune system is more or less effective at dealing with mixed- relative to single-species infections (Choisy and De Roode 2010). While I have not collected data for gametocyte densities or their sex ratios to estimate transmission potential, this could easily be obtained as samples are available. However, given that reproductive interference between different species can occur (chapter 3, Paul et al. 2002b, R. Culleton personal communication), gametocyte densities and their sex ratio may be a poor proxy for transmission success from mixed-species infections. Therefore, due to the variety of factors

involved, it is difficult (and not advisable) to make predictions about virulence evolution from single generation experiments such as the one I present in chapter 5 (Choisy and De Roode 2010). However, given that the frequency of mixed-species infections is highly variable across different geographical locations, the impact of mixed-species infections on parasite virulence could potentially be tested by quantifying virulence in single-species infections in comparable natural populations that vary in the frequency of mixed-species infections (providing variation in other factors can be controlled).

Finally, in the context of mixed infections, it is important to mention two recent studies that may potentially change some of the current views on the distribution and the nature of the interactions in mixed infections. Firstly, a study by (Portugal et al. 2011) shows that blood stage infections above a certain parasite density can impair the growth of freshly inoculated sporozoites in a density-dependent manner and that this is mediated by a hormone whose synthesis is stimulated by parasites in the blood. This is the first evidence of a form of (indirect) interference competition between malaria parasites. Second, Nkhoma et al. (2012) showed that hosts in a malaria endemic region are infected with highly related haplotypes of *P. falciparum*, which may indicate that mixed infections are acquired from the simultaneous inoculation of multiple parasite haplotypes, rather then through superinfection. Moreover, if parasites within infections are highly related, there may be more scope for cooperation between parasites than previously assumed, which could impact on patterns of virulence and disease transmission.

6.5. CONCLUSION

The work I present in this thesis reveals the importance of molecular interactions (chapter 3) and parasite phenotypic plasticity (chapter 4) for determining the outcome of transmission, demonstrates how the within-host interactions and virulence in mixed-infections can be shaped in complex ways by immunity and resource availability (chapter 5), and provides a phylogenetic context under which these processes can be understood (chapter 2). This contributes more generally towards a broader understanding of the within- and between-host biology of malaria

parasites.

A key ambition of studying the evolutionary ecology of infectious disease is to be able to explain and predict patterns of disease transmission, virulence, and recovery, and to predict parasite responses to medical interventions. However, as I think is clear from the discussion above, we still lack knowledge in a variety of fundamental processes that must be understood to achieve the above aims. Nevertheless, there are several new tools that will be essential for reaching those aims. Specifically, the "-omics" technologies, if placed in an ecological context, can help with identifying cryptic parasite phenotypes (e.g. drug tolerance) or the cues responsible for gametocyte sex determination, which could give rise to a new generation of novel and "smarter" medical interventions.

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SYNTHESIS

Plastic parasites: sophisticated strategies for survival and reproduction?

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Abstract

Adaptive phenotypic plasticity in life history traits, behaviours, and strategies is ubiquitous in biological systems. It is driven by variation in selection pressures across environmental gradients and operates under constraints imposed by trade-offs. Phenotypic plasticity has been thoroughly documented for multicellular taxa, such as insects, birds and mammals, and in many cases the underlying selective pressures are well understood. Whilst unicellular parasites face many of the same selective pressures and trade-offs, plasticity in their phenotypic traits has been largely overlooked and remains poorly understood. Here, we demonstrate that evolutionary theory, developed to explain variation observed in the life-history traits of multicellular organisms, can be applied to parasites. Though our message is general – we can expect the life-histories of all parasites to have evolved phenotypic plasticity – we focus our discussion on malaria parasites. We use an evolutionary framework to explain the trade-offs that parasites face and how plasticity in their life history traits will be expressed according to changes in their in-host environment. Testing whether variation in parasites traits is adaptive will provide new and fundamental insights into the basic biology of parasites, their epidemiology and the processes of disease during individual infections.

Understanding how natural selection shapes the traits of infectious disease causing organisms is central to evaluating the likely success of intervention strategies and virulence management (Stearns and Koella 2007). Evolutionary theory has been successfully developed as a tool for investigating the ultimate causation of biological variation in multicellular organisms, and is increasingly being applied to explain why genetic variation is maintained for traits, such as virulence, in unicellular parasites. However, other parasite life-history traits and behaviours of considerable clinical and epidemiological importance also vary extensively. Both the ultimate and proximate causes of this variation are poorly understood but available data indicate that parasites employ remarkably sophisticated strategies for maximizing their transmission success (e.g. Paul et al. 2000; Koella et al. 2002; Reece et al. 2008). In this article, we consider how evolutionary theory can be applied to explain why traits associated with growth and reproduction in malaria (*Plasmodium*) parasites vary throughout infections and in response to changes in the in-host environment. We explain the relevant evolutionary concepts, review recent success in explaining a key parasite trait (sex ratio), and outline how an evolutionary framework will provide novel and important insights into parasite biology. Our discussion focuses on malaria parasites because within-infection processes have been most thoroughly examined in this group but our message is general: the traits exhibited by parasites and pathogens are a result of natural selection optimizing resource allocation strategies.

Evolution of life history traits: trade-offs and phenotypic plasticity

Trade-offs and adaptive phenotypic plasticity are central to our evolutionary understanding of biological variation

in metazoan organisms. Fundamental to life history theory is the idea that, whilst natural selection acts to maximize some measure of fitness, the possible combinations of phenotypic traits that can be expressed are limited by trade-offs and constraints (Roff 1992). Trade-offs can take many and diverse forms, but theoreticians have most commonly considered the role of resource allocation trade-offs: organisms have limited resources to invest in the processes (e.g. growth, maintenance, and reproduction) required to successfully transmit gene copies to future generations (Roff 1992; Stearns 1992). Natural selection is expected to optimize the allocation of resources between different processes so as to maximize fitness, assuming that sufficient genetic variation exists to allow the optimum to be reached.

Understanding life history decisions and the reasons for phenotypic variability becomes more complicated when one considers that phenotypes do not evolve under constant environment conditions. The concept of adaptive phenotypic plasticity has been central to our understanding of the evolutionary consequences of environmental variation (Schlichting and Pigliucci 1998). Phenotypic plasticity is broadly defined as a change in the phenotype of a given genotype in response to a change in environmental conditions (Schlichting and Pigliucci 1998). If natural selection favours different phenotypes under different environmental conditions, we expect the evolution of plastic phenotypic responses to information or cues that predict environmental change (Gotthard and Nylin 1995; Via et al. 1995; Pigliucci 1996). Phenotypic plasticity can evolve providing that the environment is predictable and suitable genetic variation exists. Plastic responses may involve facultative changes in a single trait, whole suites of life history, behaviours, or resource allocation strategies (Stearns 1992; Pigliucci 2001).

Phenotypic plasticity is expected to be costly, in that its expression will require the diversion of resources away from other functions such as reproduction or maintenance. Costs are diverse and can include the expenditure of resources on maintaining sensory systems, gathering and processing information, or expressing alternative phenotypes. The ability to respond appropriately to changes in the environment will also be limited by organisms' ability to detect reliable cues and process the resulting information (de Witt et al. 1998). Selection will act to maximize the fitness advantages of plasticity whilst minimizing the costs involved. This trade-off could result in the evolution of a single, fixed plastic response across a population, variation in plastic responses across genotypes within a population (genotype-by-environment interactions; 'GxE'), or nonplastic, environmental specialist phenotypes (Pigliucci 2001). In the event that environmental variation is completely unpredictable, selection may instead favour the production of randomly or alternately varying phenotypes (bet hedging strategies).

Whilst individual organisms are readily identifiable as targets of natural selection in metazoans, the genotype within an infection represents the comparable target in parasites (West et al. 2006). When infections consist of a single genotype, all parasites are genetically identical and trade-off resolution will maximize the fitness of all co-infecting parasites (Hamilton 1963; Foster 2005). When infections are composed of multiple genotypes, parasites from the same genotype are more closely related to each other than to con-specifics. Each genotype could have different optimal strategies because factors such as variation in competitive ability or immune-evasion may influence the relative importance of investing in different processes.

Trait variation in the life cycle of malaria parasites

Malaria (*Plasmodium*) and related Apicomplexan parasites comprise a diverse group of pathogens that are responsible for some of the most serious infectious diseases of humans, wildlife, livestock and companion animals (Garnham 1966). Despite more than a century of research, these parasites have resisted efforts to eradicate and control them and remain responsible for between 1 and 3 million deaths per year. There are over 170 described *Plasmodium* species, which infect mammals, birds and a wide variety of reptiles (Garnham 1966). Despite their diversity, their life-cycle (Fig. 1) always includes several rounds of asexual replication in a vertebrate host and sexual reproduction in a dipteran vector. When an infected vector bites a host, infective stages (sporozoites) are released and start the pre-erythrocytic phase by invading a specific tissue. After several rounds of replication, erythrocyte-invading stages are released into the blood. These blood-stage parasites replicate asexually and disease symptoms develop (anaemia, weight loss and cerebral malaria in some cases). Every cell cycle a small proportion of asexually produced parasites develop into nonreplicating, male or female sexual stages, termed gametocytes (Smith et al. 2002). Asexual parasites do not survive when taken up by a vector in a blood meal, but the gametocytes rapidly differentiate into gametes and fertilization occurs within an hour (Micks et al. 1948; Sinden 1983a,b; Janse et al. 1986). Each male gametocyte can differentiate into up to eight gametes whereas each female gametocyte differentiates into a single gamete. To make their gametes, males must leave their red blood cells, undergo three rounds of genome replication/mitotic division, and construct eight flagella, each with a genome (Janse et al. 1986). Conversely, female gametocytes become gametes once they have condensed and left their red blood cells. Zygotes undergo a series of morphological

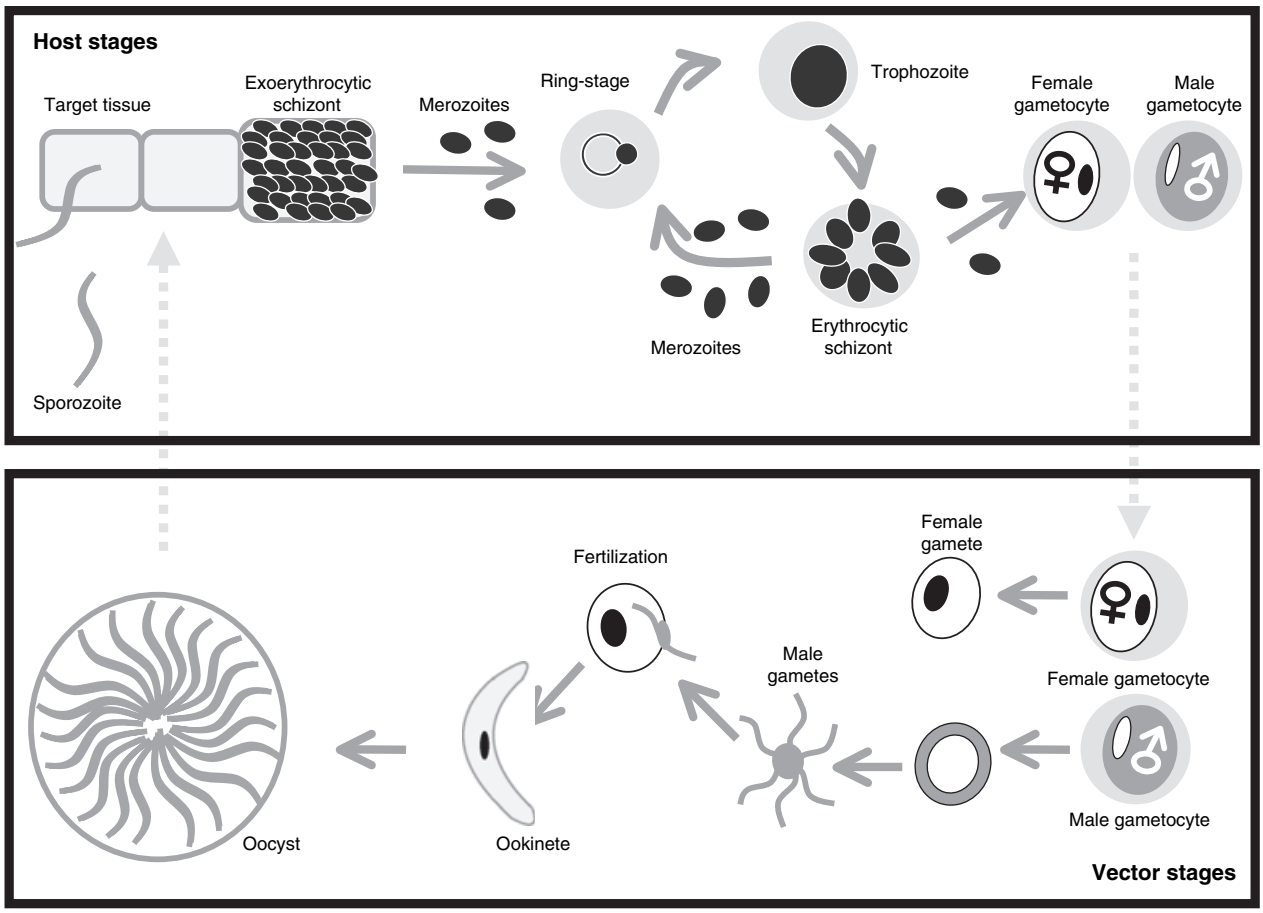

Figure 1 The life-cycle of malaria (*Plasmodium)* parasites.

transformations to establish infections in their vector, leading to the formation of thousands of host-infective sporozoites that migrate to the salivary glands ready for transmission (Baton and Ranford-Cartwright 2005).

Whilst the basic features of their life-cycle are conserved, malaria parasites exhibit considerable variation in traits associated with growth and reproduction. For example, marked variation in sex ratio patterns (Fig. 2; proportion of gametocytes that are male) occurs across species, within species, and during infections (Paul et al. 1999, 2000; West et al. 2001; Paul et al. 2002; Read et al. 2002; Paul et al. 2003; Robert et al. 2003; Reece et al. 2005, 2008). Whilst striking species differences in some traits are well documented, most have been largely overlooked. In Table 1, we highlight examples of traits for which variation has been observed between and within species and during infections. Empirical data are scarce but do show that there is interesting and important biological variation to be explained.

A fundamental question in evolutionary biology is whether or not genetic variation exists in a focal trait. Natural selection cannot produce an evolutionary response without appropriate genetic variation underlying phenotypic variation. In malaria parasites, within-species genetic variation can be tested for when genotypes (clonal isolates) can be detected (e.g. Schall and Vardo-Zalik 2007). One source of such isolates is the bank of *P. chabaudi* genotypes in the WHO Registry of Standard Malaria Parasites, held at the University of Edinburgh, UK. Experimental work has revealed that whilst these con-specific genotypes follow broadly similar infection dynamics, they vary in the maximum parasite and gametocyte densities achieved, and the degree of harm caused to their hosts in terms of weight loss, anaemia and mortality risk (Mackinnon and Read 1999; Timms et al. 2001; Mackinnon et al. 2002a,b; de Roode et al. 2003; Grech et al. 2006). Analogous field experiments using wild caught parasites of the lizard malaria (*P. mexicanum*) have also revealed evidence for genetic variation in these traits (Eisen and Schall 2000). The details of infection patterns exhibited by a given parasite genotype can also be influenced by host genotype, as observed in *P. chabaudi* (de Roode et al.

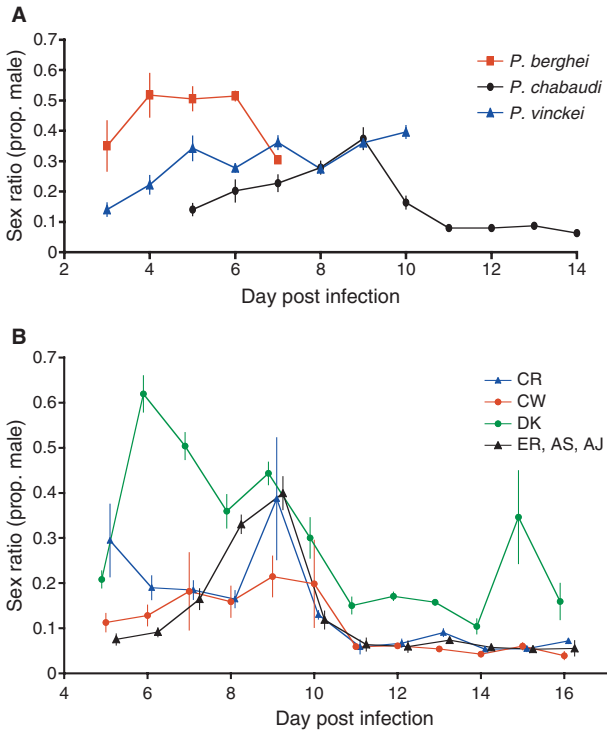

Figure 2 Sex ratios of malaria parasites vary considerably across species (A), within species (B), and during infections (A) and (B). Data shown are mean ± SE for infections of: (A) three different rodent malaria species, followed until either the infections were cleared or the experiment was terminated; and (B) six different *Plasmodium chabaudi* genotypes that follow four significantly different patterns throughout infections (Reece et al. 2008). Means are calculated from between 6 and 30 independent infections and all were initiated by 10^6 parasites in the same host genotype (MF1). Thus, this variation is not due to infective dose or host genotype.

2004; Grech et al. 2006; Raberg et al. 2007). These studies clearly show there is significant genetic variation underlying both important parasite life history traits and their expression in response to changes in the in-host environment.

Another key evolutionary question is how variation in the environment will influence selection on life history trade-offs. The 'environment' that malaria parasites experience throughout their life-cycle is remarkably complex as it encompasses the internal biotic environment of at least two other living organisms (the host and vector), and the external environment experienced by those organisms. Parasites will encounter different types of host, for which there could be different optimal solutions to resource allocation problems. During infections, the in-host environment shifts radically due to complex interactions between factors driven by both parasites and hosts (e.g. immune factors, erythropoietic state, presence of competing parasites). To thrive in such variable conditions, parasites should alter their life history decisions and behaviours to best suit each situation they encounter. The ability of natural selection to shape how parasites respond to environmental variation will depend on: the availability of reliable information on environmental conditions and the ability of parasites process it, the presence of suitable genetic variation in parasite populations (GxE), and the costs of that particular form of plasticity.

In the next section, we describe how evolutionary theory has been successfully used to understand variation in sex ratio, an important fitness-determining trait. In the subsequent sections we outline how this approach can be applied to explain other parasite life history traits that are central to survival and transmission. Whilst the concepts we present are applicable to all host and vector stages of the life-cycle, we focus on parasite traits expressed during the erythrocytic phase of infections because the necessary tools and techniques to experimentally test our predictions have recently become available.

Explaining sex ratio variation in malaria parasites

Sex allocation is one of the most well understood topics in evolutionary biology (Charnov 1982; Hardy 2002; West 2009). In many cases, simple theory can successfully predict when, why and by how much organisms should adjust their offspring sex ratio in response to a change in their circumstances. As shown in Fig. 2, sex allocation of malaria parasites varies considerably, but the application of evolutionary theory to explain this variation has been controversial (Shutler et al. 1995; Ferguson 2002, 2003; West et al. 2003).

Sex ratios in malaria parasites are generally female-biased and evolutionary theory predicts that sex ratios reflect the inbreeding rate (Read et al. 1992; Dye and Godfray 1993; West et al. 2000; Nee et al. 2002). Gametocytes taken up in a blood meal represent the genetic composition of their host's infection. The inbreeding rate is high when mating occurs between gametocytes from one or a small number of genotypes, but the inbreeding rate is low when multiple genotypes are present in a mating group. Female-biased sex ratios are expected when inbreeding occurs because this represents the most efficient allocation of resources to maximize the fertilization success of a mating group. Because each male can fertilize more than one female, a female-biased sex ratio reduces competition between related males and maximizes the number of females available to mate with (Taylor 1981). In contrast, when outbreeding, the greatest fitness returns come from increasing investment in males. In this situation, because unrelated females belonging to other genotypes are present, a genotype that produces more males

Table 1. Variation in life-history traits associated with growth and reproduction exhibited by malaria *(Plasmodium)* parasites. We highlight traits for which variation is observed across species (species), within species (genotype), and during infections (infection). This list is by no means exhaustive, but is indicative of the empirical literature to date.

Function	Trait	Examples of variation	Refs
Growth	Cell cycle duration and synchronicity	**Species**. The species of parasite infecting humans can be diagnosed from the regularity of fevers resulting from each synchronous replication cycle (*P. vivax* and *P. falciparum* for fevers every 48 h and *P. malariae* every 72 h). In the rodent malarias, *P. berghei* is asynchronous with cell cycle duration of 22–23 h, whereas mature *P. chabaudi* parasites rupture synchronously every 24 h	Mons et al. (1985), Carter and Walliker (1975), Mackinnon et al. (2002a,b), Reilly et al. (2007) and Garnham (1966)
		Genotype. *In-vitro* evidence that two clones of *P .falciparum* have an average difference of ~5 h in their cell cycle duration	
		Infection. Observations suggest that *P. chabaudi* synchronicity decreases as infections progress	
	Number of merozoites per schizont	**Species**. Species-specific data are normally presented as ranges (e.g. *P.falciparum* 8–32 merozoites per schizont), but it is not known if this represents genetic or within-infection variation. Species-specific estimates for the number of merozoites produced per schizont range from 4 to 5 in *P. juxtanucleare* (an avian malaria) to over 90 for *P. giganteum* (a lizard malaria)	Reilly et al. (2007), Garnham (1966), Kissinger et al. (2002) and Schall (1990)
		Genotype. Different clones of *P. falciparum* produce different modal numbers of merozoites per schizont, and this may also vary with the type of tissue in which parasites sequester	
		Infection. *P. berghei* produces more merozoites per schizont in reticulocytes than in mature RBC	
	Red blood cell preference	**Species**. In human malarias, *P. ovale* and *P. vivax* prefer reticulocytes, *P. malariae* prefers mature RBCs and *P. falciparum* is able to infect both age classes. Similar patterns occur in the rodent malarias; preference spans the continuum from strict preference for reticulocytes (e.g. *P. berghei*) to specialists for mature RBCs (e.g. *P. vinckei*) with generalists (e.g. *P. chabaudi*) in between. Similar examples can be found in bird malarias	Antia et al. (2008), Simpson et al. (1999), Paul and Brey (2003), Killick-Kendrick and Peters (1978) and Taylor-Robinson and Phillips (1994)
		Genotype. Some evidence that *P. chabaudi* genotypes may vary in the age range of cells they can infect	
		Infection. *P. falciparum* may become less selective as infections progress. A clone of *P. chabaudi* changes from strict preference for mature RBCs to reticulocytes around peak parasitaemia, changing again to strict preference for mature RBCs during the chronic stage of the infection	
	Cytoadherence	**Species**. Sequestration* occurs in most (e.g. *P. falciparum* and *P. berghei)* but not all (e.g. *P. malariae* and *P. knowlesii*) species investigated. Rosetting† rates vary across species, but has been observed in all species analysed	Mackinnon et al. (2002a,b), Garnham (1966), Sherman et al. (2003)
		Genotype. Genetic variation for rosetting rates observed in *P. chabaudi* (from 10% to 40%)	
		Infection. Highest rosetting rates observed before peak parasitaemia in *P. chabaudi*. Some lines of *P. falciparum* lose the ability to sequester with serial culture	

Table 1. Continued

Function	Trait	Examples of variation	Refs
Reproduction	Conversion	**Species**. Rarely calculated, potentially because data on cell cycle duration, gametocyte development time and longevity are required	Graves et al. (1984), Reece et al. (2005), Buckling et al. (1997, 1999)
		Genotype. *In vitro* data suggest genetic variation for conversion in *P. falciparum*	
		Infection. *P. chabaudi* increases conversion in response to EPO and antimalarial drugs. *P. falciparum* also increases conversion in response to antimalarial drugs	
	Sex ratio	**Species, genotype and infection**. See Fig. 2 and text (Explaining sex ratio variation in malaria parasites)	West et al. (2001), Reece et al. (2008) and Paul et al. (2000)

*Sequestration: withdrawal of infected RBCs from the peripheral circulation to the microvasculature where they adhere to endothelial cells.
†Rosetting: adherence of uninfected RBCs to infected cells leading to the formation of small clusters of cells.

will have the greatest genetic representation in the zygote population. This is 'Hamilton's Local Mate Competition' theory (LMC; Hamilton 1967) and it explains why female-biased sex ratios are favoured in spatially structured populations for a variety of taxa, from plants to insects to snakes (West et al. 2005).

The application of LMC theory to malaria parasites has proved controversial because it does not explain why sex ratios vary throughout infections or why population sex ratios in some related Apicomplexans do not correspond to their inbreeding rate (Shutler and Read 1998; West et al. 2001; Paul et al. 2003). However, cases where the data do not correspond to LMC can be explained by a simple extension of the theory, known as 'fertility insurance' (West et al. 2002; Gardner et al. 2003). Fertility insurance is important when there is a risk that not all females in a blood meal will be fertilized. This problem can arise when gametocyte density is low, due to red cell limitation in anaemic hosts or low conversion, or when transmission-blocking immune factors impair the ability of male gametocytes to make viable gametes. When male gamete viability is low or few gametocytes are able to interact in a blood meal, parasites are expected to alter their sex ratio by increasing their investment in male gametocytes, resulting in a less female-biased sex ratio than their inbreeding rate suggests. By increasing investment in males, parasites can ensure that they have produced enough males to fertilize their females.

The recent development of reverse transcription quantitative PCR assays for malaria parasites that are both genotype- and sex-specific (Drew and Reece 2007) have enabled the predictions of LMC and fertility insurance theory to be tested. Recently conducted, conceptually simple experiments in which the sex ratios produced by focal *P. chabaudi* genotypes were followed in single- and double-genotype infections, showed that parasites can evaluate their likely inbreeding rate and faculatively adjust their sex ratio according to the predictions of LMC theory (Reece et al. 2008). Furthermore, the double-infection data revealed a negative correlation between sex ratio and the proportion of gametocytes contributed by focal genotypes that quantitatively fits LMC theory. Analysis of sex ratio patterns followed throughout infections by six different *P. chabaudi* genotypes support the predictions of fertility insurance theory (Reece et al. 2008). First, female-biased sex allocation in response to LMC decreases as infections progress and parasite densities decline due to resource competition, anaemia and development of the host's immune response. Second, within-infection sex ratio patterns, for which there is significant genetic variation, can be explained by variation in anaemia and parasite densities. Third, sex ratios are adjusted in response to erythropoetin (EPO; a hormone produced by anaemic

hosts) and subsequent changes in the availability of RBC resources (Paul et al. 2000; Reece et al. 2005).

Facultative sex-ratio adjustment in response to changes in the genetic diversity of infections and within-host environment confirm that: (i) sex allocation strategies in malaria parasites are as sophisticated as those observed in multi-cellular taxa; (ii) malaria parasites can discriminate kin from non-kin and infer their own relative frequency in infections; (iii) malaria parasites are able to detect and respond to changes in host anaemia and the availability of preferred RBCs; and (iv) crucially, sex ratio variation can be explained by evolutionary theory (Shutler and Read 1998; Knowles and Sheldon 2008; Reece et al. 2008).

Adaptive plasticity in other parasite traits: keeping up with the environment?

In this section, we develop this successful application of evolutionary theory to predict how parasites should solve other trade-off problems. Specifically, we consider how plasticity in the allocation of resources to reproduction and growth should be expressed, discuss evidence for plasticity in the underlying traits, and describe how our predictions can be tested.

Investment into reproduction: conversion rate

From a life history perspective, the asexual replication of a single-genotype malaria infection can be viewed as 'growth and maintenance' and gametocyte density as 'reproduction'. As in metazoans, a fundamental trade-off exists – resources are invested into growth at the expense of reproduction and vice-versa (Stearns 1992). In metazoans, quantifying how an individual has resolved this trade-off in terms of reproductive effort is rarely possible. In contrast, the reproductive effort of malaria parasites can easily be calculated as the proportion of a cohort of parasites that develop into gametocytes (conversion rate). Though rarely measured, conversion rate frequently varies throughout infections and is increased in response to EPO and the presence of antimalarial drugs (Buckling et al. 1997, 1999; Reece et al. 2005). Here, we outline explanations for these observations and predict how parasites are expected to alter conversion in response to changes in their in-host and social circumstances. These scenarios are not mutually exclusive and in reality we expect that parasites will simultaneously be resolving many of these trade-offs.

Why should parasites increase their reproductive effort when under attack from drugs? This is a 'stressful' situation in which parasites face elimination, so the currently held belief is that parasites have nothing to lose, and should increase conversion as a last attempt at transmission before being cleared. However, theory could also predict the opposite: parasites in stressful circumstances should reduce their conversion to maximize their chances of survival (Schneider et al. 2008). In this scenario, by investing in asexual replication, parasites benefit from the future reproductive opportunities that can be attained from surviving (Mideo and Day 2008). If this is the case, why do the data show the opposite pattern? To adopt the most appropriate strategy, parasites need the ability to determine when continued survival is impossible (e.g. due to terminally decreasing parasite density or inevitable host death). For novel situations like exposure to drugs, parasites may not have been under selection for long enough to be able to evaluate their survival chances. In contrast, parasites will frequently experience severely anaemic hosts, so the ability to infer when host death is inevitable should have evolved. Available data are encouraging: *P. gallinaceum* parasites can determine whether their infections are lethal or will be resolved by hosts (Paul et al. 1999). In metazoans the analogous hypothesis (known as 'terminal investment'; Williams 1966) is very difficult to test because measurements of relative reproductive effort are required and confounding variables (e.g. decline due to senescence or infection) make it hard to measure the intrinsic state of individuals. These issues can be overcome with malaria parasites because the performance of replicate infections of genetically identical parasites can be followed in progressively deteriorating in-host conditions or drug treatment regimes.

The terminal investment hypothesis was developed from the prediction that the reproductive strategy an organism adopts will depend on the value of the fitness gains made from current reproductive effort, relative to future reproductive effort. In humans, relatively few malaria infections result in host death during the acute phase and parasites enter a longer-term (chronic) phase in which they can persist for years (Snounou et al. 2000). Because these infections have a long 'life-span', resource allocation strategies will reflect the trade-off between current and future reproductive success. More data on transmission success from acute and chronic stage infections are needed to test for this trade-off, but parasites at extremely low densities in chronic *P. falciparum* infections can infect mosquitoes, (Schneider et al. 2007).

The idea that current reproductive effort should be traded-off against future reproductive effort applies to two other situations: in-host competition and resource availability. Parasites frequently experience in-host competition with con-specific and con-generic parasites (Paul et al. 2003; de Roode et al. 2003, 2005; Mayxay et al. 2004; Bell et al. 2006). Because the best competitors are predicted to be genotypes that acquire the greatest share of RBC resources, a decrease in conversion will result in

faster replication and increased survival (Mideo and Day 2008). This prediction has been tested but the data are inconclusive: focal *P. chabaudi* genotypes monitored in single- and double-genotype infections revealed only a small reduction in conversion in the competitively inferior genotype in one of the two host strains used (Wargo et al. 2007). In addition to competition, parasites should alter their conversion when hosts become anaemic. If preferred RBCs are scarce, parasites should invest in survival, but only if their resource limitation situation is temporary. Depending on their preference for young or old RBCs, host anaemia can mean either the loss or production of preferred RBCs. In line with this prediction, *P. chabaudi,* a species that can infect young and old RBCs, increases investment in gametocytes when hosts receive EPO, but *P. vinckei,* which can only infect mature RBCs, does not alter conversion and maintains transmission by adjusting sex ratio instead (Reece et al. 2005).

Virulence and replication

Parasites must maximize survival and transmission in the face of competition and the inevitable attack of the immune response. Theory predicts that success in these endeavors is correlated with parasite virulence (damage caused to hosts) because fast growing parasites benefit from a larger pool of mature parasites to produce gametocytes. A trade-off between the rate and duration of transmission occurs because virulent parasites risk killing their host and losing future transmission opportunities (Frank 1996). However, as slow growing parasites are poor competitors and at risk from rapid clearance by the host immune response, intermediate levels of virulence are favoured by natural selection (Read 1994; Frank 1996; Day 2003; Day and Proulx 2004; Frank and Schmid-Hempel 2008). Of all parasite traits, virulence is unique in having a large body of theory but, like most traits, the data lag far behind. The idea that parasites should adjust their virulence by facultatively altering traits that underlie replication rate is both theoretically and empirically unexplored. In addition to conversion rate, plasticity in components of replication rate would also regulate virulence. Traits likely to exhibit adaptive plasticity include the number of daughter cells (merozoites) produced by mature parasites (schizonts), cell cycle duration and synchronicity, and the ability of merozoites to invade different types of RBC. In this section, we explore how parasites should adjust these traits in response to the type of host they infect and changes in their in-host environment.

The type of host that malaria parasites encounter will vary substantially in health and immune status. This variation is likely to shape how selection acts on parasite life histories. If parasites face a trade-off between growth/maintenance and reproduction, it follows that changes in conversion rate will influence replication rate and virulence. Could parasite use their conversion rate to regulate virulence? For example, reducing conversion to prioritize replication will be the best strategy when attempting to establish an infection in an adult host with an already up-regulated immune response, and with competing parasites. But, if the next host is an immunologically naïve infant, parasites will be provided with plentiful resources and no competition or immediate danger of immune attack. Uncontrolled replication in this host type will result in premature host death, and instead, we expect parasites to adopt high conversion rates to prevent host death and prolong the lifespan of their infection, with the added bonus that plenty of gametocytes result (Alizon and van Baalen 2008). Could this be the evolutionary explanation for why more gametocytes are observed in children than adults? The use of conversion rate to regulate virulence may seem intuitively unlikely because gametocytes usually represent a small (<1%) proportion of the parasites present in an infection. Indeed, a frequently posed question is 'why are there so few gametocytes?' (Taylor and Read 1997; Mideo and Day 2008). The answer may lie in the trade-off between investing resources into growth/maintenance and reproduction: the fitness benefits of increasing gametocyte number may rarely outweigh the survival costs incurred in immune hosts and in competition with con-specifics.

Alternatively, parasites could also regulate virulence by altering a key component of their cell cycle: the number of merozoites released by each mature schizont. The prerequisites for plasticity to evolve in this trait are met: there is genetic variation for the number of merozoites produced by *P. falciparum* schizonts (Reilly et al. 2007) and this trait is under fine-scale control because each nucleus within a developing schizont can divide a different number of times (Leete and Rubin 1996). Despite the vast species differences in the number of merozoites produced by schizonts, remarkably little is known about the ecological factors and evolutionary pressures shaping variation in this trait. However, increasing merozoite number would clearly be advantageous under deteriorating environmental conditions (e.g. immune attack), when survival is at risk. Data on variation in the number of merozoites in mature schizonts are extremely scarce, which is partly due to the challenges of collecting them – in many species, they sequester in tissues. New cell and molecular methods can overcome these difficulties because parasites can be collected prior to sequestration and incubated *in vitro* to maturity (Reece and Thompson 2008), or located during sequestration by whole-organism imaging methods (Franke-Fayard et al. 2006). Also, a variety of

methods can be used to identify mature schizonts (e.g. fluorescent microscopy or cell sorting), which can be prevented from rupturing by incubation with cysteine protease inhibitors (Rosenthal et al. 1987; Sijwali et al. 2004). These tools can now be used to investigate when maturing parasites commit to the number of nuclear divisions they will undertake and which factors influence this. For example, does merozoite number vary according to whether schizonts contain parasites committed to asexual or sexual differentiation? There may also be trade-offs involved between the quantity of merozoites produced and their invasion capabilities, or time required to complete a cell cycle.

In addition to variation in the duration of cell cycles, the synchronicity and development time of parasite cohorts varies substantially (Gautret et al. 1994). Conventional wisdom assumes that cell cycles are timed to match gametocyte maturation with the diurnal patterns of vectors, and synchronous development enables parasites to withstand immune attack through 'safety in numbers' (Hawking et al. 1968). Yet, these explanations are unsatisfactory because it is precisely the synchronous bursting of schizonts that elicits a rapid and short-lived immune response that sterilizes gametocytes for several hours and attacks merozoites (Naotunne et al. 1991). Such high costs to transmission suggest that synchronicity yields substantial fitness benefits or is the outcome of a constraint imposed either by host factors or parasite physiology. The latter is unlikely because parasites increase their synchronicity and developmental rate in response to host melatonin (*P. berghei;* Hotta et al. 2000) and co-culture with other parasites (*P. falciparum;* Dyer and Day 1993). Our own observations suggest that synchronicity decreases in *P. chabaudi* as infections progress. This could allow parasites to avoid attack from immune factors or be directly caused by host immunity. Experiments to distinguish between parasite and host factors are possible using mice without immune systems or by blocking the actions of specific factors. The fitness costs and benefits of synchronicity can be quantified by assaying the expression of stage specific genes in synchronous (using purified merozoites), or asynchronous (a mix of stages) infections (Mons et al. 1985). Comparing cohort development in wild type parasites with transformed parasites in which genes underlying cell cycle regulation have been deleted would also be extremely useful.

Replication rate and virulence are also determined by parasites' ability to acquire RBCs for replication. Cell and molecular data indicate there is substantial scope for adaptive plasticity in RBC preference, but variation in this trait is yet to be integrated into an evolutionary framework. The RBC preference of different species ranges from reticulocytes for *P. vivax* and *P. ovale* to mature RBCs for *P. malariae, P. falciparum* has the ability to switch its invasion strategy, and similar variation is found in the rodent malarias (Stubbs et al. 2005). Given the variation in the age and density of RBCs available in healthy and anaemic hosts during infections, the ability to facultatively alter RBC preference would be advantageous (Paul et al. 2003). The ability to invade all available RBC types may seem preferable, but different merozoite surface machinery is required for invading RBC of different ages (Cowman and Crabb 2006), and because the genes involved in invasion are also a source of antigenic variation, is it important for parasites to avoid expressing them simultaneously (Meunier 2001). This could explain why each *P. yoelii* merozoite only expresses one of the 25 genes that determine its RBC preference at a time (Preiser et al. 1999) and *P. falciparum* parasites can switch their invasion strategy (Stubbs et al. 2005). If so, invasion strategies will reflect the trade-off between the benefits of being a generalist that is able to invade a range of RBCs, versus the costs of exposing antigens to the immune system. An additional trade-off may be operating between the range of RBCs parasites can invade and their ability to grow within them. For example, *P. berghei*, parasites have a stronger preference for reticulocytes, and produce more merozoites per schizont in reticulocytes (Killick-Kendrick and Peters 1978). The molecular tools (e.g. reverse transcription quantitative PCR) required to measure gene expression and invasion abilities are available and can be applied to experiments in which host haemopoetic state is manipulated. However, to understand how parasites cope with changes in resource availability, quantitative data on RBC preference throughout infections are required. Here, mathematical models would prove particularly useful because complex erythropeotic processes determine what RBCs are available to parasites and this cannot accurately be inferred from snapshot observations of the type of RBCs that parasites have developed within (Cromer et al. 2006).

Conclusions and future directions

In addition to the specific investigations we have suggested, a substantially broader understanding of how within-infection dynamics are influenced by host factors, parasite traits, and interactions between them is required. This can be readily achieved for malaria parasites because: (i) focal genotypes can be followed during replicate infections in a range of scenarios; (ii) multiple focal genotypes can be investigated to establish whether GxE underlies patterns of trait variation and plasticity; (iii) multiple parasite and host variables can be measured and their potentially confounding influence on traits of interest can be statistically controlled for; and (iv) the use of mathemati-

cal models to examine within-host dynamics offers a new tool for hypothesis testing: iterations of theoretical developments and empirical testing promise to be a powerful approach (Mideo et al. 2008).

Evolutionary theory has been developed as a tool for investigating the ultimate causes of variation in life history traits and can provide new directions in the search for the proximate (mechanistic) processes. For example, evolutionary approaches have revealed adaptive phenotypic plasticity in sex ratio strategies, informing the search for underlying mechanistic processes (Paul et al. 2002). For other traits, such as red blood cell preference, proximate approaches have identified mechanisms likely to be involved in trade-offs and adaptive responses to environmental change. Experimental manipulations can be used to investigate what environmental information parasites use, whether these are direct or indirect cues, and what costs are paid for phenotypic plasticity. By understanding how, when and why parasites respond to different cues could enable interventions to induce parasites to make suboptimal choices that are clinically or epidemiologically beneficial. Identifying the physiological and genetic constraints on plasticity is of central importance in predicting how parasites will respond in the short- and long-term to drug and vaccine interventions. A synergy between research directed at evolutionary and mechanistic explanations for trait variation is surely the most efficient way to progress both fields.

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PLoS PATHOGENS

Sex and Death: The Effects of Innate Immune Factors on the Sexual Reproduction of Malaria Parasites

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Abstract

Malaria parasites must undergo a round of sexual reproduction in the blood meal of a mosquito vector to be transmitted between hosts. Developing a transmission-blocking intervention to prevent parasites from mating is a major goal of biomedicine, but its effectiveness could be compromised if parasites can compensate by simply adjusting their sex allocation strategies. Recently, the application of evolutionary theory for sex allocation has been supported by experiments demonstrating that malaria parasites adjust their sex ratios in response to infection genetic diversity, precisely as predicted. Theory also predicts that parasites should adjust sex allocation in response to host immunity. Whilst data are supportive, the assumptions underlying this prediction – that host immune responses have differential effects on the mating ability of males and females – have not yet been tested. Here, we combine experimental work with theoretical models in order to investigate whether the development and fertility of male and female parasites is affected by innate immune factors and develop new theory to predict how parasites' sex allocation strategies should evolve in response to the observed effects. Specifically, we demonstrate that reactive nitrogen species impair gametogenesis of males only, but reduce the fertility of both male and female gametes. In contrast, tumour necrosis factor-α does not influence gametogenesis in either sex but impairs zygote development. Therefore, our experiments demonstrate that immune factors have complex effects on each sex, ranging from reducing the ability of gametocytes to develop into gametes, to affecting the viability of offspring. We incorporate these results into theory to predict how the evolutionary trajectories of parasite sex ratio strategies are shaped by sex differences in gamete production, fertility and offspring development. We show that medical interventions targeting offspring development are more likely to be 'evolution-proof' than interventions directed at killing males or females. Given the drive to develop medical interventions that interfere with parasite mating, our data and theoretical models have important implications.

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Introduction

Malaria parasites are obliged to undertake a single round of sexual reproduction in the mosquito vector before they can transmit to new hosts, making this stage of their life-cycle a potential target for medical interventions [1,2]. The success of interventions aiming to disrupt mating success will depend upon a variety of epidemiological parameters (e.g. transmission intensity/seasonality), but will also be strongly determined by the parasites' behavioural and evolutionary responses [1–3]. Current candidates for transmission-blocking vaccines (TBV) involve targeting proteins, expressed on the surface of sexual stages, that are essential for the fertility of males (e.g. P48/45 and P230) [4–8]. However, theory predicts that the efficacy of a vaccine that reduces the fertility of one sex may be eroded if parasites respond by adjusting their sex ratios in favour of the targeted sex. The study of sex allocation has been one of the most successful areas of evolutionary biology, with empirical data matching clear theoretical predictions across a variety of taxa [9]. Before describing evolutionary theory for sex allocation strategies we outline the relevant aspects of *Plasmodium* mating biology.

Every asexual replication cycle, a small proportion of parasites differentiate into male and female sexual stages – termed gametocytes – which are developmentally arrested gamete precursors [10,11]. Gametogenesis of both sexes begins as soon as gametocytes are taken up in a mosquito blood meal, fertilization occurs within 30 minutes, and zygotes develop into the stages infective to vectors (ookinetes) after 18–20 hours [12,13]. To differentiate into gametes, gametocytes must leave the relative safety of their red blood cells (RBCs), becoming exposed to host- and mosquito-derived factors that can block mating [12]. Males are expected to be more vulnerable than females to transmission-blocking factors due to their more complex gametogenesis and mating activities [14,15]. Whereas female gametocytes only have to leave their RBCs to become gametes, male gametogenesis also includes three rounds of mitosis and flagellum construction to

Author Summary

Malaria and related parasites cause some of the most serious infectious diseases of humans, domestic animals and wildlife. To be transmitted, these parasites produce male and female sexual stages that differentiate into gametes and mate when taken up in a mosquito blood meal. Despite the need to develop a transmission-blocking intervention, remarkably little is understood about the evolution of parasite mating strategies. However, recent research demonstrates that producing the right ratio of male to female stages is central to mating success. Evolutionary theory predicts that sex ratios are adjusted in line with a variety of factors that affect mating success, including host immunity. We test this theory by investigating whether ubiquitous immune factors differentially affect the production and fertility of males and females. Our experiments demonstrate that immune factors have complex, sex-specific effects, from reducing gamete production to affecting offspring viability. We use these results to generate theory predicting how such effects shape the evolutionary trajectories of parasite sex ratio strategies. Given the drive to develop medical interventions that prevent transmission by blocking parasite mating, our results have important implications. Specifically, we suggest that medical interventions targeting offspring development are more likely to be 'evolution-proof' than interventions with sex-specific effects.

produce a (rarely achieved) maximum of eight 'sperm-like' gametes [16–20]. Mature male and female gametocytes are easily distinguished by their phenotypes as their reproductive roles result in different cellular contents [21,22]. Mature males are terminally differentiated, only having pre-synthesized proteins and machinery for gamete production (e.g. α-tubulin II, cell cycle proteins, dynein) [11,22,23]. In contrast, mature female gametocytes are prepared for continued development after fertilization, having high levels of ribosomal proteins, mitochondria (which are absent in mature males) and pools of translationally repressed messenger RNAs (mRNAs; similar to P bodies in metazoan oocytes) [11,22,24]. Therefore, male and female gametocytes are primed for gametogenesis and zygote development, respectively [25].

Sex allocation is an important fitness-related trait in *Plasmodium* and could play an important role in the response of malaria parasites to medical interventions that aim to reduce mating success [19,26–28]. Parasites could respond to transmission-blocking interventions by adjusting their sex allocation strategies via two evolutionary processes. First, if conditions within hosts are unpredictable, invariant, or if variation in within-host conditions is not a good proxy for variation in the mating conditions experienced within vectors, parasites evolve fixed (i.e. canalised) sex allocation strategies that reflect the average environment. Second, if in-host conditions reliably predict in-vector conditions, parasites will evolve to facultatively adjust their sex ratios (proportion of male gametocytes) through phenotypic plasticity. In this scenario, if asexual stage parasites detect an increase in a factor (or correlate of) that reduces mating ability in a sex-specific way, parasites will benefit from adjusting the production of male and female gametocytes in response. Given that once parasites are taken up by a vector, no further gametocyte production can occur and gametogenesis and fertilization are completed within 30 minutes, the mating environment within the blood meal is 'imported' from the host. Therefore, the within-host conditions will be good predictors for mating conditions and so facultative sex ratio adjustment is both predicted and observed [14].

Currently, two complementary evolutionary theories predict how and why parasites should adjust their investment into male and female gametocytes to maximise fertilization success. These theories – Fertility Insurance and Local Mate Competition – predict that parasites adjust sex ratios in response to environmental (e.g. transmission-blocking immunity) and social factors (inbreeding rate), respectively [14,15,29–34]. The ability of parasites to facultatively adjust their sex ratios in response to variation in the inbreeding rate has recently been verified [19,27]. Additionally, data also suggest that sex ratios are altered in response to the development of immunity [19]. Host-derived immune factors make mating challenging for parasites because they can reduce and even block fertilization [35,36]. This phenomenon, called 'transmission-blocking immunity' (TBI), has been extensively observed and documented across a variety of malaria parasite species [35–41]. The mechanisms of TBI are varied and include damaging gametocytes, preventing successful gametogenesis [36,37,41,42], decreasing the ability of gametes to interact [35,43] and preventing post-fertilization development [39,44]. Fertility Insurance predicts that when hosts mount an immune response, the fertility of male gametocytes and/or gametes is most affected, therefore parasites should produce more males to compensate [14,15]. Two lines of empirical data support this prediction. First, Paul *et al.* [26] showed that *P. gallinaceum* and *P. vinckei* increase their sex ratio in response to erythropoiesis, which is thought to act as a cue for the appearance of TBI factors. Second, Reece *et al.* [19] provided indirect support by suggesting that sex ratio variation observed during infections of different *P. chabaudi* genotypes is a mechanism to ensure fertility in face of within-host competition, host anaemia and TBI factors. Fertility Insurance currently provides the best explanation for the observed within-infection variation in the sex ratios of malaria parasites. However, the theory is based upon the untested assumption that TBI factors reduce the fertility of males more than females. Here we provide the first direct test of this key assumption by investigating whether reactive nitrogen species and pro-inflammatory cytokines, influence gametogenesis, gamete fertility and ookinete production.

Levels of reactive nitrogen species (RNS) and pro-inflammatory cytokines vary during malaria infections. These immune factors, which are ubiquitous components of the innate immune system, have been specifically implied in the sudden loss of infectivity to vectors that occurs during paroxysms and infection crisis [37,41]. Specifically, tumour necrosis factor-α (TNF-α) is a potent pro-inflammatory cytokine and several studies have revealed a role for this cytokine in mediating the killing of *Plasmodium* gametocytes, across a variety of host-parasite systems [36,41,45]. This could occur through the stimulation of phagocytosis and nitric oxide (NO) production by white blood cells [37,46,47], as these are capable of phagocytosing opsonized gametes in the mosquito midgut [48] and the inhibition of NO synthesis by white blood cells reduces in 60% the inactivation of *P. falciparum* and *P. vivax* gametocytes [37,49]. NO is produced by the enzyme inducible nitric oxide synthase in response to infection, in both hosts and vectors, and is extremely toxic at high doses. NO is a highly reactive molecule, thus a significant extent of the damage it causes is indirect, through the production of RNS (such as peroxynitrite, nitrates, nitrites or S-nitrosothiols) that frequently function as the ultimate effectors [50]. Hereafter, unless otherwise stated, we use the term 'RNS' to refer to NO and its reaction products. During *Plasmodium* infections, RNS appears to impair asexual replication, gametogenesis and zygote development [37,42,44,51]. Levels of RNS increase during *P. yoelii* infections and reduce ookinete production when either gametocytes or gametes are exposed [42].

Furthermore, RNS have been shown to induce the programmed cell death of *P. berghei* ookinetes [52] and to extensively reduce *P. berghei* oocyst burdens in *Anopheles* mosquitoes [44]. This is, at least in part, the result of a pro-inflammatory response, in which host cytokines induce the mosquito to increase NO (and therefore RNS) production [53].

Here, we use the rodent malaria parasite *Plasmodium berghei* to conduct a series of experiments to investigate how RNS and TNF-α influence mating success and ookinete production and develop theoretical models that predict the evolution of sex allocation strategies, given the effects observed in our experiments. Therefore, we use these immune manipulations as 'proof-of-principle' for other factors with similar effects on the sexual reproduction and transmission of malaria parasites. Specifically, we test whether: (1) RNS and TNF-α have dose dependent effects on male gametogenesis (exflagellation) and ookinete production; (2) exposure of male and female gametocytes to both RNS and TNF-α influences their sexual development; (3) the greater effect of RNS we observe on male gametogenesis results in sex-specific fertility effects; and (4) the observed effects of RNS depend on the developmental stage at which parasites are exposed. Our results reveal that RNS reduces male but not female gametogenesis and impairs the fertility of both sexes, whereas TNF-α only affects zygote development. The relative importance of reduced gametogenesis, impaired mating ability and reduced post-mating development have not been explicitly considered by Fertility Insurance theory. Therefore we develop a new mathematical model to derive predictions for how the effects of immune factors generated naturally or by a medical intervention are likely to impact upon parasite sex ratio evolution (a schematic of the biological effects included in the model is presented in Figure 1).

Results

All the experiments we describe below were performed *in vitro*, using gametocytes harvested form *Plasmodium berghei* infected mice. Parasites were either cultured in conditions that 'mimicked the vector' (in which they immediately became activated and underwent gametogenesis and mating; media at pH 8 and 21°C), or conditions that 'mimicked the host' (in which gametocytes remained developmentally arrested; pH 7.25, 37°C) [19]. Parasites cultured in host mimicking conditions became activated and underwent gametogenesis if subsequently exposed to vector mimicking conditions. We manipulated exposure to TNF-α with recombinant mouse TNF-α and RNS exposure with L-ana (L-Arginine p-nitroanilide dihydrochloride) and SIN-1 (3-morpholinosydnonimine hydrochloride). L-ana is an inhibitor of NO synthesis and SIN-1 donates RNS in solution (see methods for details) [54]. We exposed parasites to RNS and TNF-α treatments in 1 ml cultures with 15 or 20 µl parasitized blood.

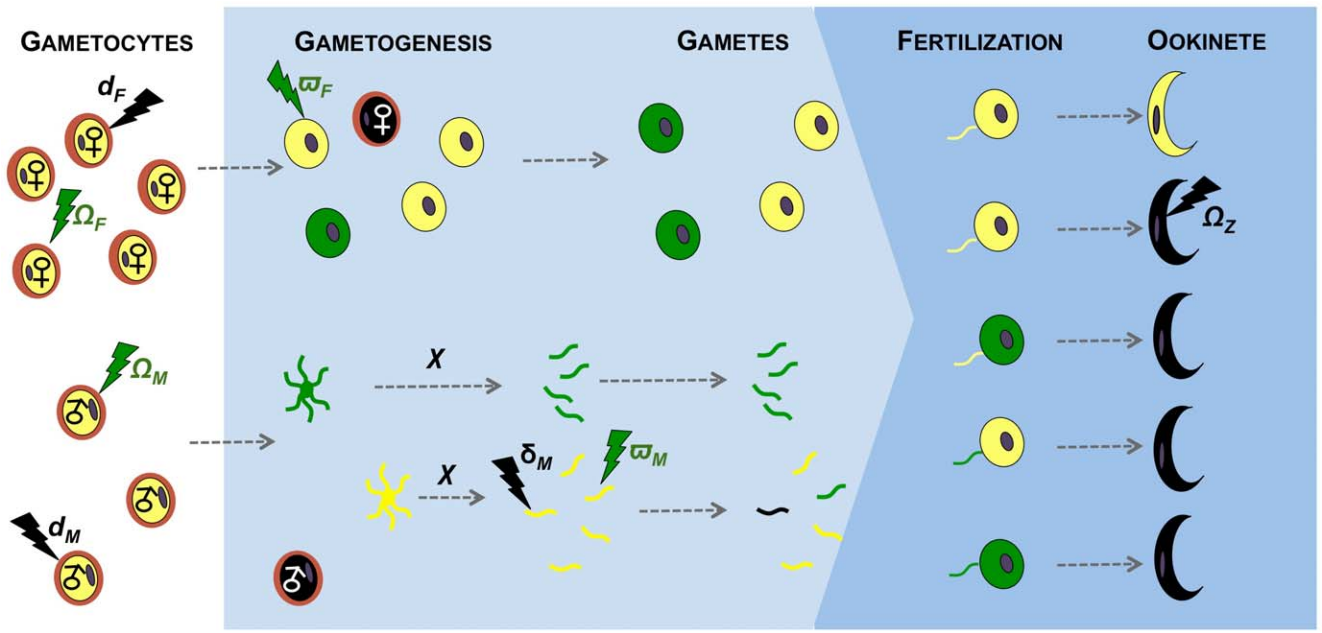

Figure 1. Effects of immunity on gametogenesis and fertility of malaria parasites. The effects of transmission-blocking immune factors on the sexual development of malaria parasites investigated in our model. Female and male gametocytes circulating in the host (white background) undergo gametogenesis when taken up by a mosquito vector (blue background). Each male gametocyte differentiates into χ gametes ($\chi \leq 8$) and each female gametocyte produces one gamete. Male gametes locate and fertilise female gametes, and the resulting zygotes develop into ookinetes. Immune factors circulating in the host can act on males and females throughout their sexual development, from gametocytes to zygotes. The developmental stages of females are shown above the stages of males and each individual gametocyte/gamete is shown in the same relative position throughout development. The effects of immune factors (lighting) on sexual stages can either be cryptic (i.e. render gametocytes/gametes dysfunctional; green), or fatal (i.e. gametocytes/gametes die; black). Healthy, unaffected, parasites are represented in yellow, dysfunctional parasites in green, and dead parasites in black. Immune factors kill female gametocytes with probability d_F and male gametocytes or gametes with probabilities d_M or δ_M, respectively. Dead sexual stages do not participate further in the mating pool. Immune factors render female gametocytes and gametes dysfunctional with probabilities Ω_F and ϖ_F respectively, and male gametocytes and gametes with probabilities Ω_M and ϖ_M, respectively. Dysfunctional gametocytes/gametes participate in the mating pool and can be fertilized as for healthy gametes, however zygotes are unviable and die before reaching the ookinete stage. Immune factors can also directly lead to zygote death with probability Ω_Z. All possible fertilization scenarios are represented: mating between two healthy gametes, mating between one healthy and one dysfunctional gamete and mating between two dysfunctional gametes.
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Experiment 1: Dose-dependent effects of RNS and TNF-α

We first tested whether RNS and TNF-α influence sexual reproduction by exposing parasites to different concentrations of these factors and assaying exflagellation and ookinete production. We incubated parasites in vector mimicking media across seven concentrations of SIN-1 (ranging from 0 to 1 mg/ml) [55] and five concentrations of recombinant mouse TNF-α (from 0 to 1 μg/ml; see Methods). Increasing concentrations of SIN-1 caused a significant linear decrease in the densities of exflagellating males ($F_{(1,35)} = 16.28$, $P<0.0001$; transformed $y = 0.16\text{-}0.10x$) and ookinetes ($F_{(1,35)} = 25.86$, $P<0.0001$; transformed $y = 0.17\text{-}0.18x$). Similarly, TNF-α also caused a significant linear decrease in the densities of exflagellating males ($F_{(1,15)} = 6.83$, $P = 0.012$; $y = 0.23\text{-}0.09x$) and ookinetes ($F_{(1,15)} = 17.53$, $P<0.0001$; transformed $y = 0.54\text{-}0.37x$).

Experiment 2: Effects of RNS and TNF-α on gametogenesis and ookinete production

Having found significant negative effects of RNS and TNF-α on exflagellation and ookinete production we investigated whether these factors interacted with each other to further reduce parasite mating success and if these effects depended on the developmental stage at which parasites were exposed (i.e. in host or vector conditions). For this set of experiments we used a fully cross-factored design, consisting of two RNS and two TNF-α levels (see Methods).

First, we investigated the effects of RNS and TNF-α on gametocytes by incubating parasites for 60 minutes in host mimicking media. We then replaced treatment media with vector mimicking media (without RNS or TNF-α manipulations) to stimulate gametogenesis and quantified the development of male and female gametocytes into gametes using the following classifications: (a) mature gametocytes still inside their RBC, (b) gametocytes that had emerged from the RBC and (c) exflagellating male gametes (see Methods for criteria). We present the proportion of a given developmental stage relative to the total number of observed gametocytes/gametes of the same sex (Figure 2). The proportion of emerged female gametocytes was not significantly influenced by either RNS ($\chi^2_1 = 2.72$, $P = 0.099$) or TNF-α ($\chi^2_1 = 0.12$, $P = 0.731$; or their interaction $\chi^2_1 = 3.38$, $P = 0.066$). In contrast, the proportion of male gametocytes that emerged from RBCs was significantly reduced by RNS ($F_{(1,\ 59)} = 81.29$; $P<0.0001$; mean 'RNS−' 0.55±0.02; 'RNS+' 0.32±0.02) but not by TNF-α ($\chi^2_1 = 0.16$, $P = 0.689$; or their interaction $\chi^2_1<0.01$, $P = 0.982$). Similarly, the ability of males to exflagellate was significantly reduced by RNS ($F_{(1,\ 59)} = 33.40$; $P<0.0001$; mean 'RNS−' 0.15±0.01; 'RNS+' 0.09±0.01) but not by TNF-α ($\chi^2_1 = 0.85$, $P = 0.36$; or their interaction $\chi^2_1 = 0.02$, $P = 0.885$).

Second, we investigated the effects of RNS and TNF-α on exflagellation and ookinete production by incubating parasites in culture media mimicking the vector environment (Figure 3). In line with the results from our previous experiments, the proportion of exflagellating males was significantly reduced by RNS ($F_{(1,\ 45)} = 11.24$, $P = 0.002$; mean 'RNS−' 0.32±0.06; 'RNS+' 0.12±0.03). This effect was enhanced by TNF-α (interaction: $F_{(1,\ 45)} = 6.67$, $P = 0.014$) but in the absence of RNS, TNF-α had no significant effect ($F_{(1,\ 45)} = 1.90$, $P = 0.175$). Conversely, the effect of RNS and TNF-α on ookinete production depended on each others presence (interaction $F_{(1,\ 24)} = 14.91$, $P = 0.001$). Specifically, ookinete production was reduced by TNF-α but only in the absence of RNS (mean 'TNF-α−' 0.41±0.06; 'TNF-α+' 0.17±0.07), whereas RNS reduced ookinete production but only when TNF-α was absent (mean 'RNS−' 0.41±0.06; 'RNS+' 0.09±0.05).

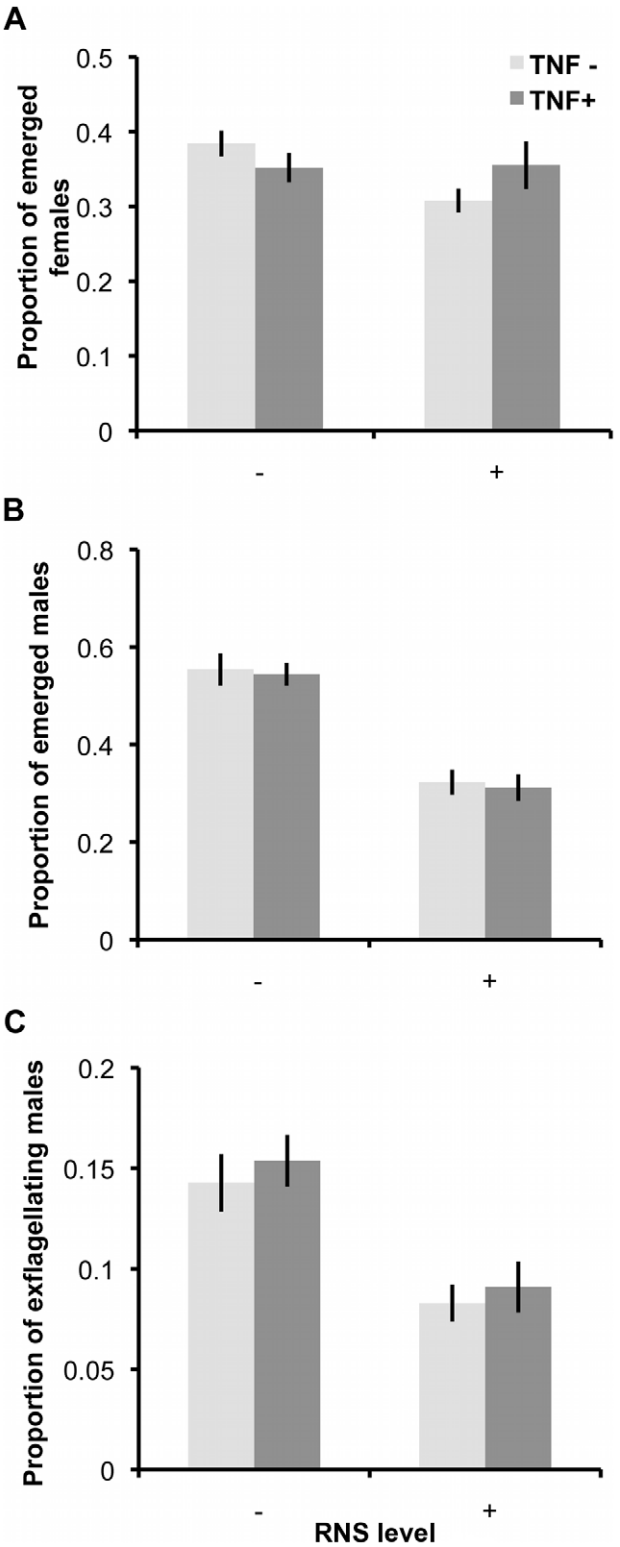

Figure 2. Ability of gametocytes to undergo gametogenesis after exposure to RNS and TNF-α. Mean (± S.E.) proportion (n = 20) of emerged female gametes (A), emerged male gametocytes (B), and exflagellating male gametes (C), relative to the total number of male or female gametocytes/gametes observed, when gametocytes are exposed to immune factors during incubation in 'host conditions' and then activated in un-manipulated 'vector conditions' media.
doi:10.1371/journal.ppat.1001309.g002

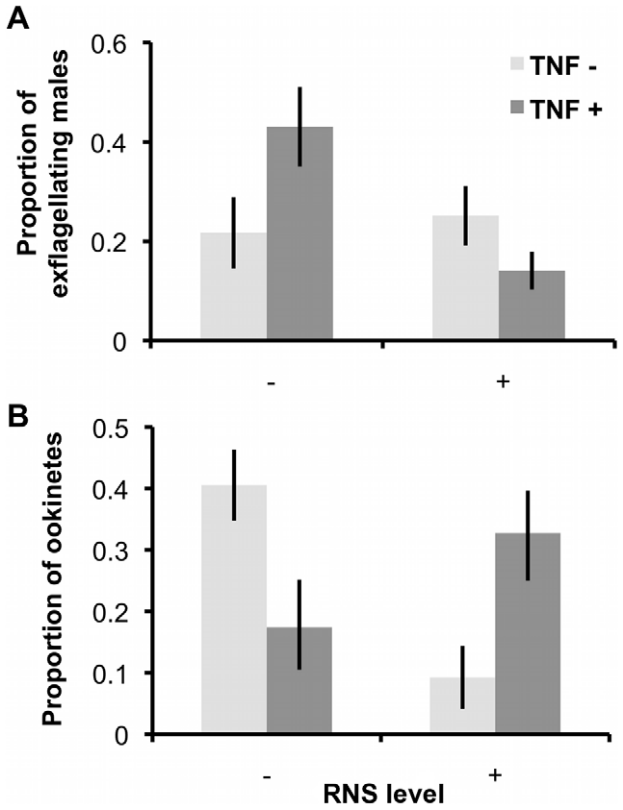

Figure 3. Exflagellation rates and ookinete production after exposure to RNS and TNF-α during gametogenesis. Mean (± S.E.) proportion of exflagellating male gametes (A; n = 16) or ookinetes (B; n = 9) produced when parasites are exposed to RNS and TNF-α during gametogenesis (in-vector conditions media). Proportions are relative to the total number of exflagellating male gametes or ookinetes produced from each infection, across treatments.
doi:10.1371/journal.ppat.1001309.g003

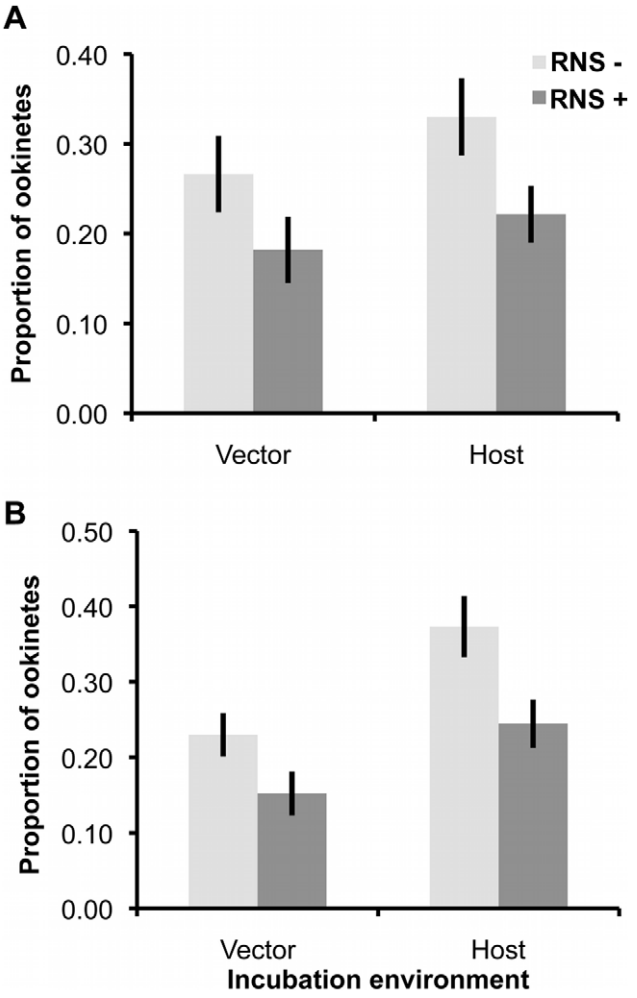

Figure 4. Ookinete production after exposure of males or females to RNS, before or during gametogenesis. Mean (± S.E.) proportion (n = 19) of ookinetes produced, when females (A) or males (B) are exposed to RNS as gametocytes (in-host conditions media) or during gametogenesis (in-vector conditions media). Proportions are relative to the total number of ookinetes produced by the focal sex from each pair of infections.
doi:10.1371/journal.ppat.1001309.g004

Experiment 3: Sex-specific effects of RNS on fertility

Experiment 2 revealed that only RNS had a significant effect on gametogenesis, in which male but not female development was impaired. Therefore, we tested whether these effects translated into sex-specific differences in fertility (i.e. whether matings with RNS exposed gametocytes/gametes resulted in fewer ookinetes), when parasites were exposed as gametocytes (in host-mimicking media) or during gametogenesis (in vector-mimicking media). We separately exposed each sex to RNS using two genetically transformed (knock-out; KO) *P. berghei* lines: Pbs48/45ko and Pbs47ko [4,6,22], which produce unviable male and female gametes, respectively. This allowed us to assay the fertility consequences of exposing one sex to RNS by providing exposed parasites with a surplus of unexposed mates from the opposite sex and assaying ookinete production (Figure 4).

We observed that RNS exposure significantly reduced fertility of both males and females regardless of whether parasites were exposed as gametocytes or during gametogenesis ($F_{(1,131)} = 15.87$, $P = 0.0001$; mean 'RNS−' 0.30 ± 0.02; 'RNS+' 0.20 ± 0.02). In contrast to our predictions, RNS did not have sex-specific effects (treatment:sex interaction: $\chi^2_1 = 0.023$, $P = 0.88$), nor was this effect influenced by exposing parasites to RNS in host- or vector-mimicking environments (treatment:environment interaction: $\chi^2_1 = 0.366$, $P = 0.55$). However, across all treatments, parasites exposed in host conditions produced significantly more ookinetes than those exposed in vector conditions ($F_{(1,131)} = 10.19$, $P = 0.0018$; mean 'Host' 0.29 ± 0.02; 'Vector' 0.21 ± 0.02).

Theoretical model

We incorporate our experimental results into Fertility Insurance theory by developing a mathematical model to explore the impact of transmission-blocking factors on the evolution of parasite sex allocation strategies. Specifically, we examine whether sex ratio adjustment could compensate for transmission-blocking factors with the following effects on males or females: preventing male or female gametocytes from undergoing gametogenesis (as each female gametocyte only produces one gamete, killing of these stages is mathematically equivalent); blocking the mating ability of male gametes; and causing damage to gametocytes or gametes such that mating can occur but zygotes are not viable. We term the latter phenomenon, of cryptic damage to gametocytes or gametes that results in a dead zygote, as dysfunction. Note that, although we do not observe all of the effects on all stages and all sexes, we incorporate them all in the model (illustrated in Figure 1),

as they are theoretical possibilities. Also, our model makes no assumptions about whether parasites evolve fixed (i.e. canalised) or facultative (i.e. plastic) sex allocation strategies.

First, we show that all zygote mortality effects (i.e. treatments leading to $0<p<1$) have no impact on the evolutionarily stable (ES) sex ratio [56,57]. We write $W=\zeta(z)\,p$, i.e. fitness is the product of zygote production and zygote viability, where zygote production depends upon sex ratio but zygote viability does not. The direction of selection is given by the derivative of fitness with respect to sex ratio [58], and this 'marginal fitness' is $\mathrm{d}W/\mathrm{d}z=(\mathrm{d}\zeta/\mathrm{d}z)p$. The ES sex ratio z^* satisfies $\mathrm{d}W/\mathrm{d}z|_{z=z^*}=0$, i.e. selection does not favour an increase or decrease in sex ratio when the population is at the ES sex ratio, and this is equivalent to the condition $\mathrm{d}\zeta/\mathrm{d}z|_{z=z^*}=0$ for all $p>0$. Since ζ is not a function of p, it follows that z^* is not a function of p (and hence is not a function of Ω_Z, Ω_M, Ω_F, ϖ_M or ϖ_F; see Methods and Figure 1 for symbol definitions). Therefore, treatments that simply impact upon the viability of zygotes (e.g. cause gametocyte/gamete dysfunction) are not expected to have an evolutionary impact upon parasite sex ratios.

Second, to investigate the impact of model parameters arising from gametocyte or gamete killing on the ES sex ratio, we write an explicit expression for expected fitness:

$$W=\sum_{\alpha=0}^{q}\sum_{\Gamma=0}^{\alpha}\sum_{\gamma=0}^{\chi\Gamma}\sum_{\phi=0}^{q-\alpha}\binom{q}{\alpha}z^{\alpha}(1-z)^{q-\alpha}\binom{\alpha}{\Gamma}{d_M}^{\alpha-\Gamma}(1-d_M)^{\Gamma}\binom{\chi\Gamma}{\gamma}\delta_M{}^{\chi\Gamma-\gamma}(1-\delta_M)^{\gamma}\binom{q-\alpha}{\phi}{d_F}^{q-\alpha-\phi}(1-d_F)^{\phi}\min\{\gamma,\phi\}p \quad (1)$$

The condition $\mathrm{d}W/\mathrm{d}z|_{z=z^*}=0$ can be solved numerically for z^* for any numerical parameter set (q, d_M, d_F, δ_M). An exploration of the ES sex ratio z^* across this parameter space is presented in Figures 5 and S1, S2, S3. Specifically, we recover the prediction that the gametocyte ES sex ratio will be biased towards the more limiting sex when factors prevent male or female gametocytes from undergoing gametogenesis or block the mating ability of male gametes.

Discussion

Evolutionary theory developed to explain the sex allocation strategies of metazoan taxa has enjoyed huge success. Recently, there has been growing interest in whether this theory could be applied to protozoans, particularly malaria parasites [14]. The sex ratios of malaria parasites are normally female biased, but extensive variation occurs during the course of infections [27]. Evolutionary theory offers an explanation for this variation and predicts that in-host conditions will influence parasite sex allocation strategies if host-derived immune factors disproportionately reduce the fertility of males relative to females [14,15,29–34]. Here, we tested this assumption by quantifying the effects of two well-known innate TBI factors (RNS and TNF-α) on sexual development and fertility of malaria parasites [15,31]. We show that: (1) RNS and TNF-α reduce the densities of exflagellating males and ookinetes in a dose-dependent manner; (2) TNF-α can reduce ookinete densities, but only in the absence of RNS (Figure 3); (3) RNS impairs male but not female gametogenesis (Figure 2 and 3), and reduces the fertility of both males and females independently of whether parasites are exposed as gametocytes or during gametogenesis (Figure 4). We then explored the consequences of our results for parasite sex ratio evolution, by incorporating them into Fertility Insurance theory (Figures 1 and 5) [15,31]. Specifically, our model demonstrates that the ES sex ratio will be biased towards the sex that has a lower number of surviving gametes reaching the mating pool and that the extent of this bias increases as the number of gametocytes in the mating group (q) increases. We also show that factors causing gametes to become dysfunctional (resulting in inviable zygotes) do not affect the ES sex ratio. Below, we discuss the results of our experiments, explain the evolutionary predictions of our model and its implications for the development of transmission-blocking interventions.

RNS, TNF-α and the sexual development of malaria parasites

In our experiments, RNS reduced male but not female gametogenesis while impairing the fertility of both sexes. How can these results be explained? In parasitic infections, high levels of RNS may cause: oxidative damage of DNA (leading to mutations and strand brakes); inhibition of DNA repair and synthesis; inhibition of protein synthesis; inhibition of mitochondrial activity; down- or up-regulation of cytokine (e.g. TNF-α) levels [50,59]. As described in the introduction, male and female gametocytes are prepared for gametogenesis and zygote development respectively [25]. If RNS can impair DNA synthesis and/or microtubule assembly, males would not be able to produce gametes. In contrast, female gametogenesis does not involve these activities and females 'simply' need to leave their RBCs, for which they use the contents of pre-synthesized secretory organelles called osmiophilic bodies [60]. Therefore, whilst female gametogenesis and mating *per se* is unlikely to be influenced by RNS, the development of fertilized females into zygotes and ookinetes is likely to be affected. For example, damage to stored mRNA and inhibition of protein synthesis or mitochondrial activity (e.g. cytochrome oxidases) would impair meiosis (at ~3 h after fertilization) and zygote development, but not impair fertilization [18,50,59]. These effects could explain the observed results, because instead of reducing the ability of females to differentiate into gametes, the effects of RNS would be expressed after fertilization (which we term dysfunction) and lead to female-dependent zygote death, resulting in fewer ookinetes. Here we did not identify the causal RNS and their relative contributions. However, this will be important if transmission-blocking interventions cause or mimic the activities of RNS.

Our experiments show that TNF-α consistently reduces ookinete production and whilst we observed a reduction in exflagellation in some experiments, this effect was inconsistent. Why does TNF-α reduce ookinete production? As TNF-α functions are mainly modulatory and need time to develop, it is possible that gametogenesis and mating occur before the effects of TNF-α manifest. Ookinete development takes about 18–20 hours from fertilization and during this time TNF-α could exert its effects, which could also involve the activation of apoptotic-like death [61,62]. Recent experiments provide support for our interpretations, as the deletion of genes coding for proteins essential for the storage and stabilization of translationally repressed mRNAs, in female gametocytes/gametes, do not reduce fertilization success, but substantially reduce the differentiation of zygotes into ookinetes [24,63]. Interestingly, deletion of different genes can affect zygotes throughout development, suggesting that damage to stored mRNA could abort zygote development at multiple stages (e.g. before or after meiosis) [24].

Evolution of parasite sex allocation strategies: Theoretical predictions

The results of our experiments show that TBI factors can affect the sexual development and fertility of male and female parasites

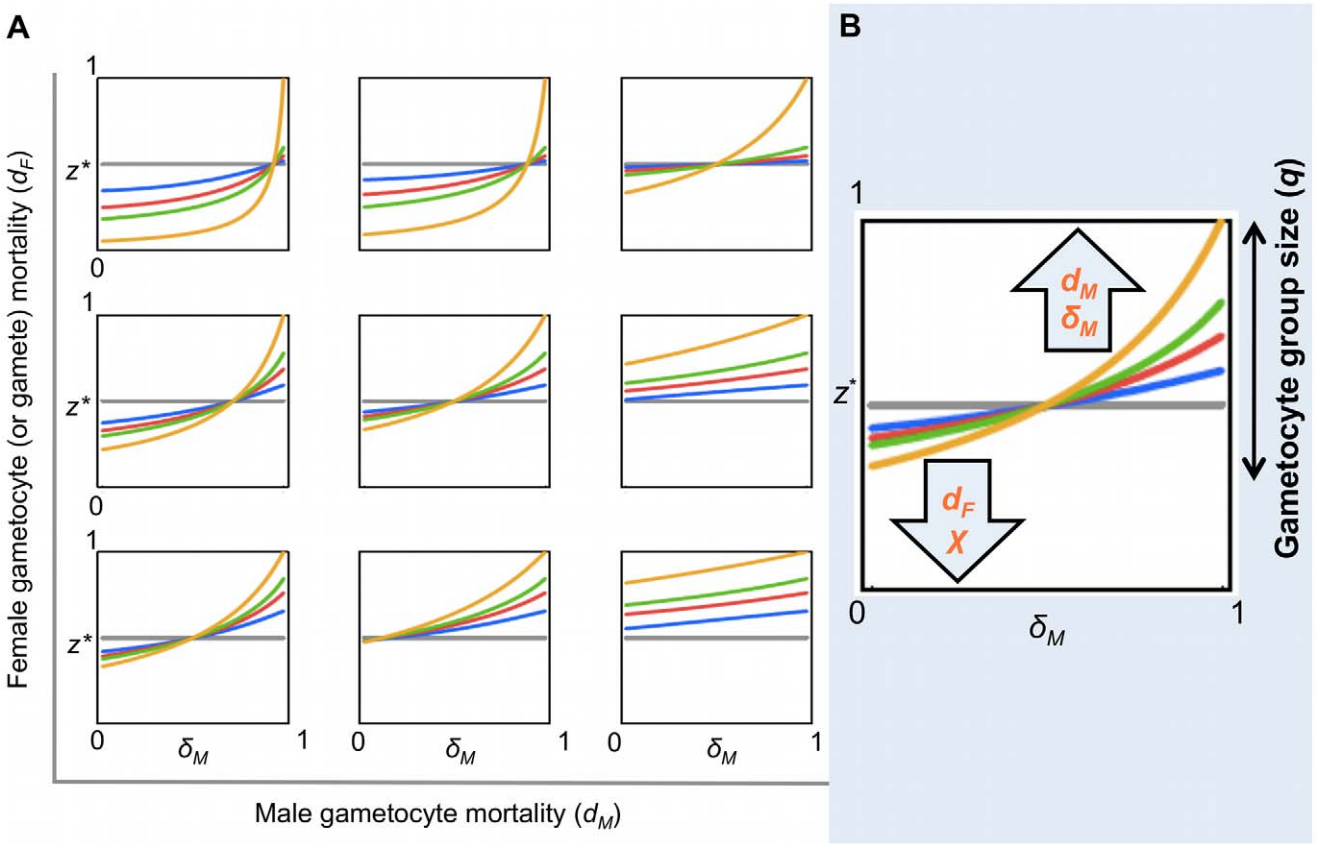

Figure 5. Evolutionarily stable sex allocation strategies when sex- and stage-specific mortality rates vary. Effect of male and female gametocyte mortality and male gamete mortality on the ES gametocyte sex ratio (z^*), for a clonal population, when the number of gametes produced per male gametocyte (χ) is 2 (this fecundity has been estimated for this system by other studies; see ref. [19]). Figures S1, S2, S3 show similar patterns to Figure 5A for $\chi = 1$; 4; 8, respectively. (A) For each plot within the panel, z^* varies with male gamete mortality rate (δ_M). The coloured lines represent different gametocyte group sizes (q): 2 (grey), 5 (blue), 10 (red), 20 (green) and ∞ (yellow). Each plot depicts different parameter combinations of male gametocyte ($d_M = 0.1$; 0.5; 0.9) and female mortality rate ($d_F = 0.1$; 0.5; 0.9), with d_M increasing left to right and d_F increasing bottom to top. (B) Cartoon summarizing the effects observed in Figures 5A and S1, S2, S3. The set of possible values for z^* is strongly influenced by q. The number of gametes of each sex reaching the mating pool (which depends on the mortality parameters and on χ) influences z^* within the constraints determined by q. Within each plot, the effects of δ_M and q on z^* can be clearly observed: the magnitude of sex ratio change increases with q and z^* increases to compensate for higher δ_M. The effects of d_M and d_F can be observed by comparing the points where the lines cross the y axes (i.e. $\delta_M = 0$) across the plots: z^* increases along rows with increasing d_M and decreases up the columns with increasing d_F. The effect of χ on z^* can be observed by comparing plots that are in the same position in different figures: sex ratio becomes more female biased as χ increases.
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and that the stage at which this occurs is sex-specific. As illustrated in Figure 1, we incorporated the observed and potential effects of transmission-blocking factors on males and females, at all stages of development, into Fertility Insurance theory and generated new predictions for the evolution of parasite sex allocation strategies. Our model predicts that the ES gametocyte sex ratio will be insensitive to variation in gametocyte or gamete dysfunction and zygote mortality. This means that treatments that impact upon the viability of zygotes are not expected to have an evolutionary impact upon parasite sex ratios. In contrast, we predict that the best (ES) sex ratio strategy will vary depending on an interaction between gametocyte group size (q), number of gametes formed per male gametocyte ($0 \leq \chi \leq 8$) and gamete and/or gametocyte mortality. Although, our model makes no assumptions about whether parasites achieve an ES sex ratio through the evolution of facultative or fixed sex allocation strategies, facultative sex allocation is predicted for reasons already outlined in the introduction.

In the context of clonal infections, the ES sex ratio maximises the expected number of viable zygotes, i.e. maximises the expected number of gametes of the minority sex present in the mating pool (this excludes dead gametocytes/gametes, but includes dysfunctional gametocytes/gametes). For an infinite gametocyte group size (i.e. $q \rightarrow \infty$), that behaves deterministically, the ES sex ratio is one that leads to the same number of male and female gametes being present in the mating pool. This is the sex ratio z^* that satisfies $cz^* = 1\text{-}z^*$, i.e. $z^* = 1/(c+1)$, where c is the number of male gametes, able to mate, produced per male gametocyte [15,32]. Thus, the ES sex ratio is female biased if $c > 1$, and male biased if $c < 1$ (Figures 5 and S1, S2, S3). However, for finite mating groups ($q < \infty$) – that behave stochastically – the expectation of mating success must be calculated over the whole distribution of possible outcomes. This will tend to reduce the extent to which the sex ratio is biased towards the sex favoured in the deterministic case [15,31]. For example, in the extreme of a gametocyte group size of two ($q = 2$; the lowest mating group size for which mating success is possible), the ES sex ratio is always $z^* = 0.5$ (regardless of other parameter values), to maximise the probability of both sexes being present (Figures 5 and S1, S2, S3). Additionally, we reveal that, in a small portion of parameter space – corresponding to very small

gametocyte group sizes, low female mortality, and high male gametocyte mortality and fecundity (χ) – fertility insurance can even lead to a sex ratio bias in the opposite direction (i.e. producing a female biased sex ratio, despite the risk of the absence of males in the mating pool; Figures S2 and S3). This non-intuitive result is due to the way stochastic variation in the number of gametocytes of each sex alters the variance as well as the expected number of gametes of each sex that reach the mating pool. Although the conditions under which this occurs are restrictive, they may be met in natural infections, as many individuals carry gametocytes at extremely low densities [64]. In the context of our experiments and assuming parasites can facultatively adjust sex ratios, our model predicts that if q is high enough to allow for sex ratio adjustment, then RNS should induce parasites to increase the production of male gametocytes.

Our data suggest that RNS reduced female fertility by rendering gametocyte/gametes dysfunctional, so that their fertilisation results in the production of unviable zygotes. The reduction in ookinete production by TNF-α could also be due to male or female dysfunction or, more likely, through increasing zygote mortality. Therefore, we examined the influence of gametocyte and gamete dysfunction and zygote mortality on the evolution of parasite sex allocation strategies. We found that the ES gametocyte sex ratio is independent of these factors (i.e. the occurrence of gametocyte/gamete dysfunction and zygote mortality does not change the relative fitness of different sex ratio strategies). Put simply, this suggests that zygote mortality or gametocyte/gamete dysfunction will not impose selection on parasite sex allocation strategies as parasites cannot compensate for the loss of reproductive success through sex ratio adjustment. More broadly, other immune factors, such as antibodies or complement, could also impair the sexual reproduction of malaria parasites and the effects of such factors should be easily interpreted in light of our theoretical models.

To bring our mathematical modelling in line with our experiments we have focused on the importance of mortality and dysfunction throughout the sexual development of malaria parasites. However two additional factors have an important impact in sex allocation strategies of malaria parasites: (1) the inbreeding rate and (2) the rate at which asexually replicating parasites commit to gametocyte production (conversion rate). The effect of inbreeding on the ES sex ratio is well understood, with theory (Local Mate Competition) enjoying strong empirical support [14,19,29,32–34]. For clonal mating groups, the ES sex ratio strategy is the one that maximises the overall mating success of the infection as the parasites behave as a single, unified decision maker [14,27]. In contrast, in mixed infections, conflicts between clones occur, such that the ES sex ratio is the one that maximises each individual clone's inclusive fitness and not the overall mating success of the infection [14,27]. But for the work we present here, extending our model to allow for a finite number of independent clones per host would not change the qualitative results we present. Fertility Insurance theory predicts that if a low conversion rate results in a small number of gametocytes being taken up by the vector (i.e. small q), parasites should produce a less female biased sex ratio than expected by the inbreeding rate alone. This is due to the stochastic risk of too few males being present in the blood meal to fertilize the females when sex ratios are female biased [15]. One intuitive solution for this would be to produce more gametocytes. However, given that gametocyte production comes at a cost to asexual replication, parasites face a trade-off between investment in in-host survival and reproduction (i.e. transmission). Increasing gametocyte conversion is a solution that will not always be available and might be impossible when parasites are 'stressed' (e.g. by in-host competition and low doses of anti-malarial drugs) [65,66]. Therefore, if transmission-blocking interventions also affect asexual stages and reduce in-host survival, parasites are likely to reduce conversion rates and produce fewer gametocytes.

Implications for transmission blocking interventions

Our model reveals that an intervention with a sex-specific effect on mating ability will elicit an evolutionary response. However, sex ratio adjustment cannot fully rescue zygote production, given that an increase in the number of male gametocytes comes at the cost of decreasing the number of female gametocytes. Nevertheless, in a scenario of widespread transmission-blocking vaccination or treatment with gametocidal drugs with a sex-specific effect, natural selection will "compare" the fitness of parasites that do, and do not, adjust their sex allocation strategies, leading to an increase in the frequencies of the former. Therefore, quantifying the impact of sex ratio adjustment on rescuing fertility and thus, fitness is now required. In contrast, our model also reveals that a transmission-blocking factor resulting in zygote mortality or gametocyte/gamete dysfunction will be 'evolution proof' with respect to parasite sex allocation strategies. Therefore, we suggest that current efforts to prevent fertilization by targeting proteins with sex-specific phenotypes, such as P230, P48/45 (involved in gamete attachment) or Pfg377 (female emergence from the RBC), will be less effective than vaccines targeting zygote development (e.g. P28) [5,60,67]. An alternative transmission-blocking approach could cause dysfunctional female gametes by targeting the expression of female-specific translationally repressed mRNAs [24]. Furthermore, a transmission-blocking intervention combining targets for gamete dysfunction and zygote death would minimize possible redundancy effects, which have been observed in several knock-outs of malaria parasites (e.g. P48/45) [6].

Conclusions

Given the drive to develop transmission-blocking interventions that disrupt sexual reproduction in malaria parasites, there is an urgent need to evaluate how their short- and long-term success will be influenced by parasite mating strategies. Here, we combined experiments with mathematical modelling to predict how transmission-blocking factors influence parasite sex allocation strategies. Our model predicts that transmission-blocking interventions causing gametocyte/gamete dysfunction and/or zygote mortality will be 'evolution-proof' from the perspective of imposing selection on parasite sex ratio strategies, i.e. parasites may still evolve other strategies or traits to cope with a transmission-blocking intervention, but these will have to be independent of sex allocation. Put simply, understanding the behavioural strategies that parasites have evolved to cope with naturally occurring transmission-blocking immune factors, will inform predictions for how they will respond to a transmission-blocking factor. More broadly, understanding how, when and why parasites respond to changes in their in-host environment will facilitate the development of interventions that induce parasites to make decisions that are suboptimal for their transmission success, but that are clinically or epidemiologically beneficial. For efficient progress, synergy between research directed at evolutionary and mechanistic explanations for parasite traits and strategies is required.

Methods

Hosts and parasites

We maintained MF1 mice, aged 8–10 weeks (Harlan-Olac, UK; or in house supplier, University of Edinburgh), on *ad libitum* food (RM3(P), DBM Scotland Ltd, UK) and water (supplemented with

0.05% PABA to enhance parasite growth), with a 12 hour light cycle, at 21°C. We initiated infections by intra-peritoneal inoculation of 10^7 parasitized RBCs in 100 µl carrier consisting of 50% Ringers (27 mM KCl, 27 mM $CaCl_2$, 0.15 M NaCl), 47.5% heat-inactivated foetal bovine serum and 2.5% heparin (5 units ml^{-1}). For experiments 1 and 2, we inoculated female mice, previously (day −3 or −4) treated with 60 mg/kg of phenylhydrazine (PHZ), with *P. berghei* line 820 [68]. For experiment 3 we inoculated male mice (PHZ treatment: 125 mg/Kg, day −2) with one of two *P. berghei* KO lines: Pbs48/45ko or Pbs47ko [4,6,22]. We treated mice with PHZ because the resulting release of young RBCs increases gametocyte production in *P. berghei*, which maximises the number of gametocytes that can be harvested for *in vitro* mating experiments [69]. For each experiment, parasites were collected from mice on day 3 or 4 post-infection, and each infection contributed parasites to all treatments to control for any potentially confounding influences of differences between infections.

Animal ethics statement

All the protocols involving mice passed an ethical review process and were approved by the U.K. Home Office (Project License 60/3481). Work was carried according to the Animals (Scientific Procedures) Act, 1986.

Culture conditions

In order to manipulate the levels of RNS and TNF-α we used the following chemicals: recombinant mouse TNF-α (Sigma, UK), L-ana (Sigma, UK) and SIN-1 (Sigma, UK). We dissolved all chemicals in phospate buffered saline and exposed parasites to treatments in 1 ml cultures with 15 or 20 µl parasitized blood. L-ana is a specific inhibitor of the activity of the enzyme inducible nitric oxide synthase which becomes active in response to infection. SIN-1 donates NO and/or superoxide, in solution, at different rates depending on the specific conditions in which SIN-1 is incubated [54,70,71]. However, given that superoxide and NO react with each other at an extremely fast rate to produce peroxynitrite ($ONOO^-$), SIN-1 is likely to act as a donor of either NO or peroxynitrite, depending on the rates at which SIN-1 generates NO and superoxide [54]. The oxygen concentration of the solution is one of the major determinants of whether SIN-1 behaves as a NO or peroxynitrite donor, donating mostly NO in anaerobic conditions and peroxynitrite in aerobic conditions [54]. In our cultures, oxygen concentrations were in-between fully anaerobic and aerobic conditions, as parasites were incubated in closed 1.5 ml tubes. Biological agents, such as human plasma or heme proteins, which are similar to components of our cultures (e.g. mouse plasma, haemoglobin) increase the capacity of SIN-1 to donate NO [54]. Furthermore, as peroxynitrite can react to produce several RNS (e.g. nitrite, nitrate, S nitrosothiols or nitrosyl-metal complexes) and as we did not measure the specific contributions of each of these factors, we use the term RNS to refer to the factors present in cultures exposed to SIN-1 [50,61,72]. We did not measure RNS and TNF-α levels in our cultures for three reasons. First, our focus is on testing the effects of RNS and TNF-α on the sexual development of parasites. As our experiments were designed so that each host contributed blood and parasites to all treatment groups in a given experiment, this controls for any variation between infections and ensures that our results are due to the RNS and TNF-α manipulations each culture was subjected to. Second, TNF-α levels were directly manipulated with recombinant mouse TNF-α. Third, we are not aware of any method that would allow us to measure total levels of the different RNS in small volume cultures.

Experiment 1

We set up cultures with vector mimicking media for the following SIN-1 concentrations: 0, 0.00001, 0.0001, 0.001, 0.01, 0.1 and 1 mg/ml [55], with 6 mice contributing parasites to each treatment. We tested the following concentrations of recombinant mouse TNF-α: 0, 0.005, 0.01, 0.5 and 1 µg/ml with 4 mice contributing parasites to each treatment. We recorded the densities of exflagellating males after 15–20 minutes and ookinetes after 18–20 hours using a haemocytometer.

Experiment 2

We used the following RNS and TNF-α levels: 1 mg/ml SIN-1 (RNS+), 1 mg/ml of L-ana (RNS−), and presence (TNF-α+) or absence (TNF-α−) of 1 µg/ml recombinant mouse TNF-α. Parasites from each of 20 mice were exposed to all four combinations of treatments. We used the following criteria to classify developmental stages of gametogenesis after 15 minutes incubation in vector mimicking media: (1) Mature gametocytes: still inside their RBC; females have blue-purple cytoplasm, small, well defined purple nucleus surrounded by a small nucleolus; males have pink-yellow cytoplasm and large disperse pale-pink nucleus. (2) Emerged females: female gamete condensed into a more circular shape, without a vacuole, cytoplasm staining a more intense blue and a less obvious nucleolus than in a female gametocyte. (3) Emerged male: male gamete with a large circular nucleus in the centre of the cell surrounded by a ring of cytoplasm. (4) Exflagellating male: emerged male gamete progressed to forming up to 8 flagella that protrude from the cell and stain red-purple [73–75]. We also recorded the densities of exflagellating males and ookinetes as described for experiment 1.

Experiment 3

We infected 38 mice with Pbs47ko (n = 19) or Pbs48/45ko (n = 19). We set up mating cultures following Reece et al. [19], by pairing infections according to proximity of their sex ratios, calculated from the densities of Pbs48/45ko female gametocytes in giemsa stained smears (using criteria described for Experiment 2) and Pbs47ko exflagellating males (as for Experiment 1). To avoid pseudo-replication, each infection was only used in 1 pair. For each pair of mice, we made 8 sets of 1 ml cultures, either with (RNS+) or without (RNS−) 1×10^{-5} mg/ml SIN-1, mimicking host (60 min. incubation) or vector conditions (15 min. incubation), to which we added 15 µl of parasites from one of the infections in each pair. These single sex cultures provided 'exposed' parasites for fertility testing, and corresponded to the following factorial design: 2 conditions (host/vector)×2 SIN-1 exposures (RNS+/−)×2 sexes (male/female). After incubation we replaced media in all cultures with 1 ml vector mimicking media (without any SIN-1 manipulation). While 'exposed' parasites were incubating, we collected 60 µl of blood from each infection's pair and added these 'unexposed' parasites to 4 ml cultures in vector mimicking media (without SIN-1). Each 1 ml culture of the 'exposed' parasites was then added to a 4 ml culture containing its 'unexposed' pair and incubated to produce ookinetes (as for Experiment 1). This allowed us to ensure that the mating success of the 'exposed' sex would not be limited by the availability of 'unexposed' gametocytes from the opposite sex. All the cultures were timed so that 'exposed' parasites were added to the cultures containing their 'unexposed' mates at the same developmental stage. For example, a final 5 ml culture could contain 15 µl of blood from a RNS exposed Pbs48/45ko infection (in which females are the 'exposed' sex) and 60 µl of blood from a Pbs47ko infection (in which ~4 times more males are provided as 'unexposed' mates). We also set up cultures in vector mimicking

media to verify that 'unexposed' parasites from each line are unable to produce ookinetes on their own. We recorded the densities of ookinetes as described for experiment 1.

Statistical analysis

We used linear mixed effects models (R version 2.7.0; The R Foundation for Statistical Computing; www.R-project.org) because, by treating each infection (or pair of infections in Experiment 3) as a 'random' effect, we can account for problems associated with pseudoreplication arising from repeated measurements of each infection. In order to meet the assumptions made by parametric tests we arcsine square root transformed response variables where necessary. We minimised models following stepwise deletion of the least significant term and using log-likelihood ratio (χ^2) tests to evaluate the change in model deviance until only significant terms remained, and we present F-ratios for fixed effects remaining in minimal models. We then re-ran minimal models using restricted maximum likelihood to estimate the effect sizes reported in the text. Unless otherwise indicated, data and estimated effect sizes are presented as proportions of the focal parasite stage produced in a given treatment, relative to that produced across all treatments for each infection.

Theoretical model

We assume an infinite host population, divided into infected and uninfected individuals, with infected hosts containing a single infection producing haploid gametocytes that circulate in the blood. We assume that q gametocytes are transferred from host to vector during blood feeding, and that these gametocytes form a single mating group. The expected proportion of males in the mating group is z, i.e. the sex allocation strategy of the parasite strain that contributed the gametocytes. Hence, the actual number of males is a random variable $\alpha \sim Bi(q,z)$ (i.e. binomially distributed with q trials and probability of success z). Consequently, the number of female gametocytes is q-α. Male and female gametocytes are killed with probability d_M and d_F respectively, leaving $\Gamma \sim Bi(\alpha, 1-d_M)$ surviving males and $\phi \sim Bi(q-\alpha, 1-d_F)$ surviving females. We assume every surviving male produces χ gametes, and every surviving female produces a single gamete. We consider that male gametes are killed with probability δ_M, and hence $\gamma \sim Bi(\chi\Gamma, 1-\delta_M)$ male gametes enter the mating pool. We assume that all ϕ female gametes enter the mating pool (death of female gametes is formally equivalent to that of female gametocytes, and hence is implicitly included in the parameter d_F). Therefore, the number of zygotes is equal to the number of gametes of the limiting sex, i.e. $\zeta = min(\gamma, \phi)$. Finally, we assume that only a proportion p of zygotes are viable, due to either: (a) factors that kill each zygote with probability Ω_Z; (b) factors acting on gametocytes resulting in the production of dysfunctional gametes at rate Ω_M for males and Ω_F for females; or (c) factors acting on gametes and causing them to become dysfunctional at rate ϖ_M for males and ϖ_F for females, i.e. $p = (1-\Omega_Z)(1-\Omega_M)(1-\Omega_F)(1-\varpi_M)(1-\varpi_F)$. In this context, we use the term 'dysfunctional' to refer to a gamete that achieves fertilisation but carries sufficient damage to render the resulting zygote inviable (i.e. unable to develop as an ookinete). Inviable zygotes will result when one or both of the parental gametes are dysfunctional. Hence, the number of viable zygotes produced by the mating group is $W = \zeta p$, and this is our measure of fitness [15,31,32].

Supporting Information

Figure S1 Evolutionarily stable sex allocation strategies when sex- and stage-specific mortality rates vary ($\chi = 1$). Effect of male and female gametocyte mortality and male gamete mortality on the ES gametocyte sex ratio (z*), for a clonal population, when the number of gametes per male gametocyte (χ) is 1. On each plot, z* varies with male gamete mortality rate (δM). The coloured lines represent different gametocyte group sizes (q): 2 (grey), 5 (blue), 10 (red), 20 (green) and ∞ (yellow). Every plot depicts different parameter combinations of male gametocyte (dM = 0.1; 0.5; 0.9) and female mortality rate (dF = 0.1; 0.5; 0.9), with dM increasing left to right and dF increasing bottom to top.
Found at: doi:10.1371/journal.ppat.1001309.s001 (0.16 MB PDF)

Figure S2 Evolutionarily stable sex allocation strategies when sex- and stage-specific mortality rates vary ($\chi = 4$). Effect of male and female gametocyte mortality and male gamete mortality on the ES gametocyte sex ratio (z*), for a clonal population, when the number of gametes per male gametocyte (χ) is 4. On each plot, z* varies with male gamete mortality rate (δM). The coloured lines represent different gametocyte group sizes (q): 2 (grey), 5 (blue), 10 (red), 20 (green) and ∞ (yellow). Every plot depicts different parameter combinations of male gametocyte (dM = 0.1; 0.5; 0.9) and female mortality rate (dF = 0.1; 0.5; 0.9), with dM increasing left to right and dF increasing bottom to top.
Found at: doi:10.1371/journal.ppat.1001309.s002 (0.21 MB PDF)

Figure S3 Evolutionarily stable sex allocation strategies when sex- and stage-specific mortality rates vary ($\chi = 8$). Effect of male and female gametocyte mortality and male gamete mortality on the ES gametocyte sex ratio (z*), for a clonal population, when the number of gametes per male gametocyte (χ) is 8. On each plot, z* varies with male gamete mortality rate (δM). The coloured lines represent different gametocyte group sizes (q): 2 (grey), 5 (blue), 10 (red), 20 (green) and ∞(yellow). Every plot depicts different parameter combinations of male gametocyte (dM = 0.1; 0.5; 0.9) and female mortality rate (dF = 0.1; 0.5; 0.9), with dM increasing left to right and dF increasing bottom to top.
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Author Contributions

Conceived and designed the experiments: RSR SER. Performed the experiments: RSR LC. Analyzed the data: RSR SER. Wrote the paper: RSR JA LC AG SER. Developed the theoretical models: JA AG.

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